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Review

Recent Advances in the Innate Immunity of Invertebrate Animals

Sadaaki Iwanaga and Bok Luel Lee†,*

The Chemo-Sero-Therapeutic Research Institute, Okubo 1-6-1, Kumamoto 860-8568, and
Department of Biology, Kyushu University, Fukuoka 812-8581, Japan
†College of Pharmacy, Pusan National University, Jang-Jeong Dong, Kum-Jeong Ku, Pusan 609-735, Korea

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Invertebrate animals, which lack adaptive immune systems, have developed other systems of biological host defense, so called innate immunity, that respond to common antigens on the cell surfaces of potential pathogens. During the past two decades, the molecular structures and functions of various defense components that participated in innate immune systems have been established in Arthropoda, such as, insects, the horseshoe crab, freshwater crayfish, and the protochordata ascidian. These defense molecules include phenoloxidases, clotting factors, complement factors, lectins, protease inhibitors, antimicrobial peptides, Toll receptors, and other humoral factors found mainly in hemolymph plasma and hemocytes. These components, which together compose the innate immune system, defend invertebrate from invading bacterial, fungal, and viral pathogens. This review describes the present status of our knowledge concerning such defensive molecules in invertebrates.

Keywords: Ascidian, *Bombyx mori*, Crayfish, Defense molecules, *Drosophila*, *Holotrichia diomphalia*, Horseshoe crab, Innate immunity, Invertebrate animals, *Manduca*, *Sarcophaga*, *Tenebrio*

Introduction

The innate immune system is the first line of inducible host defense against bacterial, fungal, and viral pathogens (Hoebe *et al.*, 2004). This defense system is essential for the survival and perpetuation of all multicellular organisms (Hoffmann *et al.*, 1999; Salzet 2001). Invertebrates, which do not possess immunoglobulins, have developed unique modalities to detect and respond to microbial surface antigens like lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, peptidoglycan (PGN) and (1 → 3) β-D-glucans (Begum *et al.*, 2000). Because both invertebrates and vertebrates respond to these substances, it is likely that a system recognizing these epitopes emerged at an early stage in the evolution of animals (Medzhitov and Janeway, 2000; Aderem and Ulevitch, 2000). Moreover, it is well known that various microbial cell wall components elicit a variety of responses that depend on species and cell type (Hoffmann *et al.*, 1999; Cooper *et al.*, 2002). Table 1 summarizes the major biological host defense systems of invertebrates; such systems are also found in mammals (Aderem and Ulevitch, 2000). In invertebrates, toll-like receptor-mediated antimicrobial peptide production (Lemaitre *et al.*, 1996; Imuler and Hoffmann, 2000; Krutzik *et al.*, 2001; Underkill and Orinsky, 2002), hemolymph coagulation (Iwanaga *et al.*, 1978), melanin formation (Sugumaran, 2002), and lectin-mediated complement activation (Fujita, 2002) are prominent immune responses. In addition to these enzyme cascades, a variety of agglutinin-lectins and reactive oxygen-producing and phagocytic systems cooperate with immune reactions to kill invading pathogens (Bogdan *et al.*, 2000). Figure 1 shows the principal defense systems associated with phagocytosis. Invaders detected by these systems are ultimately

Table 1. Major host defense systems in invertebrate animal

1. Hemolymph coagulation system
2. Pro-phenoloxidase (pro-PO) activating system
3. Lectin-complement system
4. Agglutinin-lectin system
5. Antibacterial, antifungal, and antiviral systems mediated by Toll-like receptors and peptidoglycan binding protein (PGBP)
6. Reactive oxygen-producing system
7. Phagocytic system

Abbreviations: lipopolysaccharides, LPS; peptidoglycan, PGN; C-reactive proteins, CRPs; Limulus intracellular coagulation inhibitors, LICI; prophenoloxidase, pro-PO; PGN recognition protein-LC, PGRP-LC; immune deficiency, IMD.

*To whom correspondence should be addressed.
Tel: 82-51-510-2809; Fax: 82-51-513-2801
E-mail: brlee@pusan.ac.kr

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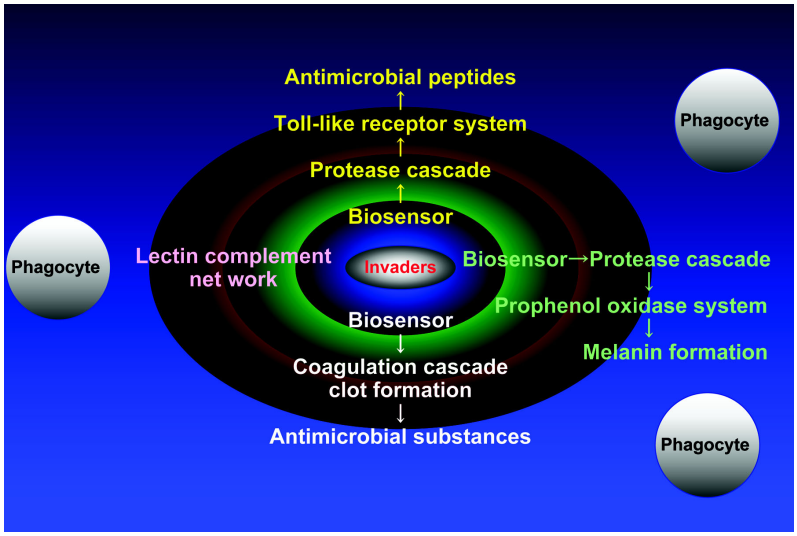

Fig. 1. The principal host defense systems associated with phagocytosis in invertebrates. The major innate immune systems include; hemolymph coagulation, melanization mediated by phenoloxidase, the expression of antimicrobial peptides mediated by Toll-like receptors and the immuno deficiency (IMD) pathway, and the lectin/complement pathway mediated by bacterial cell wall components. Invaders detected by these systems are ultimately engulfed by phagocytic cells, such as macrophage-like, neutrophil-like, or dendrotic cells, and then internalized, processed, and killed. Please refer to the following recent reviews for more information about these systems: Muta and Iwanaga, 1996, 1998; Inamori *et al.*, 2004; Hoffmann et al, 1999, Cerenins and Söderhäll, 2002; Fujita, 2002; Azumi, *et al.*, 2003; Greenberg and Grinstein, 2002; Theopold *et al.*, 2002, 2004; Kim *et al.*, 2002; Iwasaki and Medzhitov, 2004; Hultmark, 2003; Ashida and Brey, 1998; Kanost *et al.*, 2004; Natori, *et al.*, 1999; Nonaka, 2001; Nellaiappan and Sugumaran, 1996; Cooper *et al.*, 2002.

engulfed by phagocytes, such as macrophage-like, neutrophil-like and dendritic cells, and are then internalized as phagosomes and finally killed (Greenberg and Grinstein, 2002).

Here, we focus on the present status of innate immunity in invertebrates, with an emphasis on the structures and functions of the defense molecules found the following; the horseshoe crab, the freshwater crayfish, ascidians, and insects including *Drosophila melanogaster, Sarcophaga peregrina, Bombyx mori, Manduca sexta, Holotrichia diomphalia* and *Tenebrio molitor* (Fig. 2).

Horseshoe crab (*Tachypleus (T) tridentatus*)

Hemolymph and circulating hemocytes The invertebrate *Tachypleus tridentatus* (Japanese horseshoe crab) (Fig. 2A) is an arthropod, and relies completely on innate immunity, by employing unique and highly efficient host defense systems (Iwanaga *et al.*, 1992). The hemolymph plasma of this animal contains many soluble defense molecules, such as hemocyanins, various lectins, and C-reactive proteins, and thioester bond containing proteins (α_2-macroglobulins), in addition to a large numbers of granular hemocytes (amebocytes), which undergo a rapid degranulation on contact with pathogens (Iwanaga *et al.*, 1998). Hemocytes, which compose more than 99% of circulating cells, contain a variety of defense molecules, which are located in two types of secretary granules large (L)-and small (S)-granules (Fig. 3) (Muta and Iwanaga, 1996; Iwanaga and Kawabata, 1998). L-granules selectively store more than 25 defense components with molecular masses between 8 and 120 kDa. These include clotting factors, a clottable protein coagulogen, proteinase inhibitors, lectins, and antimicrobial proteins. In contrast, the S-granules contain at least six antimicrobial peptides and several proteins of molecular mass <30 kDa. These peptides include large amounts of hairpin-like tachyplesin (17-18 amino acid residues, >10 mg per individual), tachystatins (41-44 residues), tachycitins (73 residues) and big defensins (79 residues), which are highly active against Gram-negative and -positive bacteria and fungi (Iwanaga *et al.*, 1994; Iwanaga, 2002).

Table 2 summarizes the various proteins and peptides identified to date in *T. tridentatus* hemocytes and hemolymph plasma. Compared with mammalian blood the hemolymph plasma of *Tachypleus tridentatus* contains relatively few protein types (>300 different proteins are present in human plasma), and instead contains three predominant protein types, namely, hemocyanin (O_2 transporter), C-reactive proteins (CRP) (Iwaki *et al.*, 1999), and α_2-macroglobulins (Iwaki *et al.*, 1996; Armstrong, 2001; Husted *et al.*, 2002). Moreover, circulating hemocytes are extremely sensitive to bacterial LPS, and respond by degranulating a number of granular components after LPS-mediated stimulation, which results in the formation of hemolymph clot. This rapid clotting response is believed to be important for the animal's host defense, which involves

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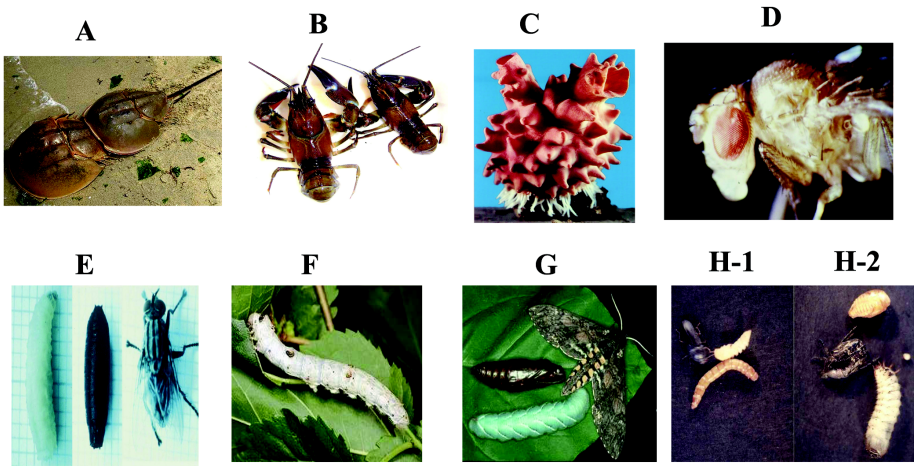

Fig. 2. Mating Japanese horseshoe crabs (*Tachypleus tridentatus*) (A), the freshwater crayfish (*Pacifastacus leniusculus*) (B), the sonitary ascidian (*Halocynthia roretzi*) (C), *Drosophila* (D), Salcophaga (E), *Bombyx mori* (F), *Manduca sexta* (G), *Tenebrio molitor* (H-1) and *Holotrichia diomphalia* (H-2).

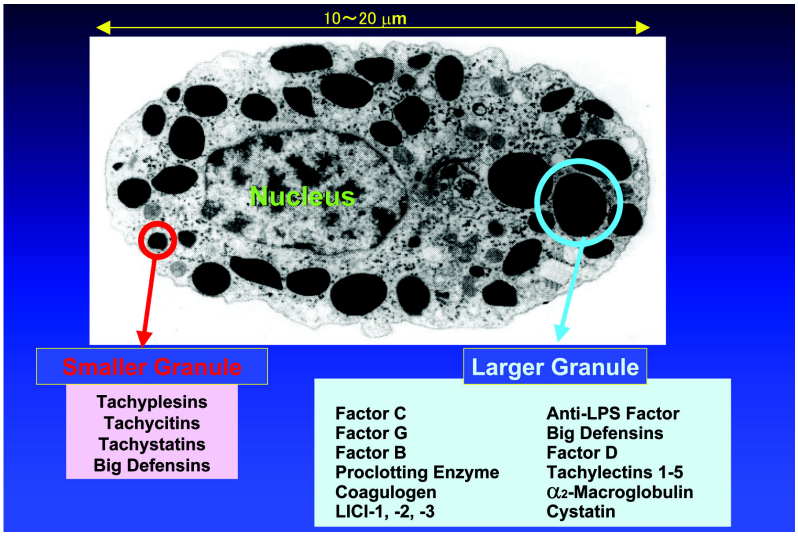

Fig. 3. Electron micrograph of horseshoe crab (*T. tridentatus*) hemocytes, and major defense molecules that have been identified in large and small cell granules.

engulfing of invading microbes, and in addition prevents hemolymph leakage (Muta and Iwanaga, 1996).

Hemolymph clotting system The hemolymph-clotting phenomenon was first identified as a prominent defense system in the horseshoe crab (*Limulus polyphemus*) by Bang (Bang, 1956). When Gram-negative bacteria invade the hemolymph, hemocytes detect LPS molecules on their surfaces (Ariki *et al.*, 2004), and then release, via rapid exocytosis, the contents of L- and S-granules (Iwanaga, 1993a,b). These released granular components include two biosensors, named factors C and G (Table 2). These two factors are serine protease zymogens and are autocatalytically activated by LPS or (1 → 3)-β-D-glucan, which are major components of the cell walls of Gram-negative bacteria and fungi, respectively. In 1996, Tamura *et al.* reported that hemocytes contain a (1 → 3)-β-D-Glucan binding protein, which differs from factor G as it does not participate in the hemolymph clotting cascade. One of the authors of this review has previously described in detail LPS and (1 → 3)-β-D-glucan-mediated clotting cascades and their molecular structures, and the functions of the five clotting factors (Table 2), factor C, factor G, factor B, proclotting enzyme, and clottable coagulogen, which all participate in clotting cascades

Table 2. Defense molecules found in hemocytes and hemolymph plasma of the horseshoe crab

Proteins and peptides	Mass (kDa)	Function/specificity	Localization
Coagulation factors			
Factor C	123	Serine protease	L-granule
Factor B	64	Serine protease	L-granule
Factor G	110	Serine protease	L-granule
Proclotting enzyme	54	Serine protease	L-granule
Coagulogen	20	Gelation	L-granule
Protease inhibitors			
LICI-1	48	Serpin/factor C	L-granule
LICI-2	42	Serpin/clotting enzyme	L-granule
LICI-3	53	Serpin/factor G	L-granule
Trypsin inhibitor	6.8	Kunitz-type	ND
LTI	16	New type	ND
LEBP-PI	12	New type	L-granule
Limulus cystatin	12.6	Cystatin family 2	L-granule
α_2-Macroglobulin	180	Complement	Plasma & L-granule
Chymotrypsin inhibitor	10	ND	Plasma
Antimicrobial substances			
Anti-LPS factor	12	GNB	L-granule
Tachyplesins	2.3	GNB, GPB, FN	S-granule
Polyphemusins	2.3	GNB, GPB, FN	S-granule
Big defensin	8.6	GNB, GPB, FN	L & S-granule
Tachycitin	8.3	GNB, GPB, FN	S-granule
Tachystatins	6.5	GNB, GPB, FN	S-granule
Factor D	42	GNB	L-granule
Lectins			
Tachylectin-1	27	LPS (KDO), LTA	L-granule
Tachylectin-2	27	GlcNAc, LTA	L-granule
Tachylectin-3	15	LPS (O-antigen)	L-granule
Tachylectin-4	470	LPS (O-antigen), LTA	ND
Tachylectin-5	380-440	*N*-acetyl group	Plasma
Limunectin	54	PC	L-granule
18K-LAF	18	Hemocyte aggregation	L-granule
Limulin	300	HLA/PC, PE, SA, KDO	Plasma
LCRP	300	PC, PE	Plasma
TCRP-1	300	PE	Plasma
TCRP-2	330	HLA/PE, SA	Plasma
TCRP-3	340	HLA/SA, KDO	Plasma
Polyphemin	ND	LTA, GlcNAc	Plasma
TTA	ND	SA, GlcNAc, GalNAc	Plasma
Liphemin	400-500	SA	Hemolymph
Carcinoscorpin	420	SA, KDO	Hemolymph
GBP	40	Gal	Hemolymph
PAP	40	Protein A	Hemolymph
$(1 \rightarrow 3)\beta$-D-glucan binding protein	168	Pachyman, cardlan	Hemocyte
Others			
Transglutaminase (TGase)	86	Cross-linking	Cytosol
8.6 kDa protein	8.6	TGase substrate	L-granule
Pro-rich proteins (Proxins)	80	TGase substrate	L-granule
Limulus kexin	70	Precursor processing	ND
Hemocyanin	3600	O_2 transporter (PO activity)	Plasma
Toll-like receptor (tToll)	110	ND	Hemocyte
L1	11	Unknown	L-granule
L4	11	Unknown	L-granule

LICI, *Limulus* intracellular coagulation inhibitor; LTI, *Limulus* trypsin inhibitor; LEBP-PI, *Limulus* endotoxin-binding protein-protease inhibitor; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; FN, fungus; LPS, lipopolysaccharide; LAF, *Limulus* 18-kDa agglutination-aggregation factor; KDO, 2-keto-3-deoxyoctonic acid; PC, phosphorylcholine; PE, phosphorylethanolamine; SA, sialic acid; TTA, *Tachypleus tridentatus* agglutinin; LCRP, *Limulus* C-reactive protein; TCRP, *Tachypleus* C-reactive protein; HLA, hemolytic activity; LTA, lipoteichoic acid; GBP, galactose-binding protein; PAP, protein A binding protein; PO, phenoloxidase; ND, not determined.

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(Iwanaga *et al.*, 1992; Iwanaga 1993a,b; Muta and Iwanaga 1996a,b). Therefore, we will only provide an outline of the clotting cascades here. Figure 4 illustrates the LPS and (1 → 3)-β-D-glucan-mediated clotting cascades of the hemolymph of *T. tridentatus* (Iwanaga *et al.*, 1994), and includes limulus intracellular coagulation inhibitors (LICI), which act as regulators of the cascade reaction (Agarwara *et al.*, 1996). These clotting cascades both involve four serine protease zymogens, factors C (123 kDa), B (64 kDa), G (110 kDa), proclotting enzyme (54 kDa), and coagulogen (20 kDa) (Bergner *et al.*, 1996, 1997). In the presence of LPS or synthetic lipid A analogs, factor C is autocatalytically activated to an active form, factor $\overline{C}$ (Iwanaga *et al.*, 1992; Tan *et al.*, 2000). Factor B zymogen is then activated by factor $\overline{C}$ to its active form (factor $\overline{B}$), which activates proclotting enzyme to clotting enzyme. Clotting enzyme then converts coagulogen to an insoluble coagulin gel, which is composed of non-covalent homopolymers, through head to tail interaction (Kawasaki *et al.*, 2000). On the other hand, factor G zymogen consisting of two heterosubunits and is autocatalytically activated in the presence of (1 → 3)-β-D-glucan, in the absence of any other protein (Muta *et al.*, 1995). The resulting active factor $\overline{G}$ activates proclotting enzyme directly, resulting in coagulin gel formation (Takaki *et al.*, 2002). Recently, Osaki *et al.*, (2002) found that non-covalent coagulin homopolymers are cross-linked by bridging hemocyte cell surface proteins, named proxins (Table 2), in the presence of hemocyte-derived transglutaminase (Tokunaga *et al.*, 1993; Osaki and Kawabata, 2002). This indicates that cross-linking is important at the final stage of hemolymph clotting to facilitate hemostasis and wound healing, as has been reported in the mammalian blood clotting system (Iwanaga, 1993a,b).

Interestingly, the NH_2-terminal portions of zymogen factor B and of proclotting enzyme contain a small compact domain containing three disulfide bonds, called the “clip domain” (Muta and Iwanaga, 1996). A similar “clip domain” has also been reported in the NH_2-terminal proenzyme regions of *Drosophila*-derived serine proteases, as described later. Moreover, the folding pattern of the three disulfide bridges located in the “clip domain” is identical to that of “big defensin”, which was recently identified as an antimicrobial peptide in *T. tridentatus* hemocytes. As the COOH-terminal end of the “clip domain” in proclotting enzyme constitutes a hinge region susceptible to proteolysis, the “clip domain”, in the same manner as defensin, might be released during the activations of serine protease zymogens, in order to act as an antimicrobial substance. In fact, the “clip domain” derived from the prophenoloxidase activated serine protease of freshwater crayfish has an antimicrobial activity similar to that of human β-defensin (Wang *et al.*, 2001). Thus, the clotting cascade could also produce antimicrobial agents, and thus provide a dual action clotting and killing system against invaders (Krem and Docera, 2002; Theopold *et al.*, 2004).

As described later in detail, the prophenoloxidase activation system found in insects (Ashida and Brey, 1998) and

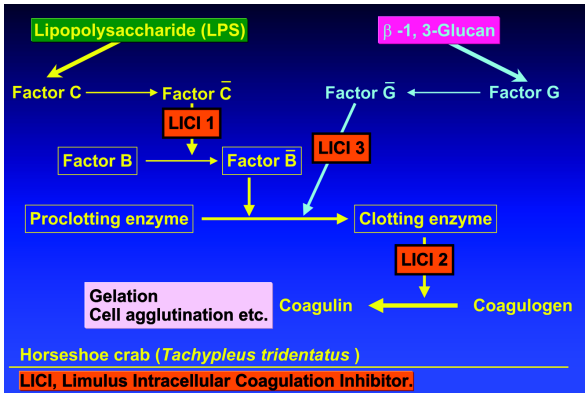

Fig. 4. LPS- and (1 → 3)-β-D-glucan mediated clotting cascades found in horseshoe crab (*T. tridentatus*) hemocytes. LICI, limulus intracellular coagulation inhibitor. This figure depicts the biochemical principle of the so called limulus test, which is used for detecting bacterial endotoxins. The method was developed by Levin and Bang (1964) based on a finding that a trace amount of endotoxin coagulates the hemocyte (amebocyte) lysate of the American horseshoe crab, *Limulus polyphemus*. This gelation reaction has been widely employed as a simple and highly sensitive assay for endotoxins (LPS). The limulus test is dependent on the protease cascade reaction shown in the figure, and is being used extensively in combination with new technology (Iwanaga *et al.*, 1978, Tanaka and Iwanaga, 1993, Obayashi *et al.*, 1985).

crustaceans is an important aspect of innate immunity, and functions to detect and kill invading pathogens and to produce melanin and its derivatives to encapsulate invaders and facilitate wound healing (Fig. 1). Recently, Decker and Tuczek reported that hemocyanin of the tarantula *Eurypelma californicum* expresses phenoloxidase activity after limited proteolysis with trypsin and α-chymotrypsin (Decker and Tuczek, 2000). Furthermore, hemocyanins of the crab *Carcinus maenas* and the lobster *Homarus Americanus* also express significant phenoloxidase activity in the presence of perclorate. Moreover, the phenoloxidase activities of the hemocyanins of the horseshoe crab *Limulus polyphemus* (Nellaiappan and Sugumaran, 1996) and *T. tridentatus* are induced in the presence of sodium dodecylsulfate and phosphatidylethanolamine (Sugumaran, 2002). Nagai and Kawabata (2000) also found that the clotting cascade of *T. tridentatus* is linked to prophenoloxidase activation, with the oxygen carrier hemocyanin functioning as a prophenoloxidase substitute. Active clotting enzyme or factor $\overline{B}$ (active form) functionally transform hemocyanin to phenoloxidase, and this conversion plateaus at a stoichiometry of 1 : 1 without proteolytic cleavage. Interestingly, proclotting enzyme also induces hemocyanin based phenoloxidase activity, but neither factors C nor G zymogens have any effect on hemocyanin. Although the functional roles of these links between the clotting cascade and hemocyanin-based phenoloxidase systems *in vivo* is not clear, these results suggest that

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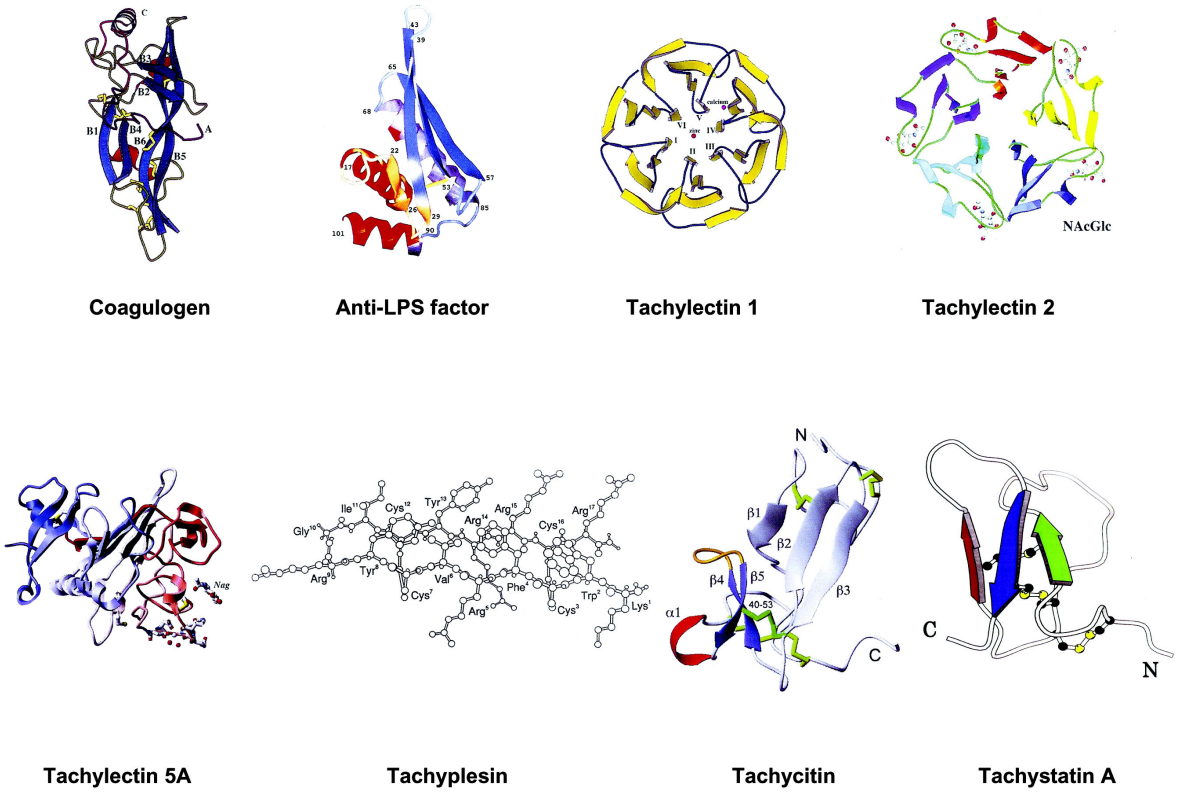

Fig. 5. The 3D structures of major defense molecules isolated from *T. tridentatus* hemocytes and hemolymph plasma. Coagulogen, tachylectins 1 and 2, and anti-LPS factor are stored in L-granules. The antimicrobial peptides, tachyplesin, tachycitin, and tachystatin A are located in S-granules. Tachylectin 5A is mainly found in hemolymph plasma.

hemocyanin exists abundantly in hemolymph plasma and that it may participate in the innate immune system of the horseshoe crab (Nagai *et al.*, 2001).

Lectin-agglutinin system The innate immune system of the horseshoe crab also recognizes invading pathogens by using a combinatorial method involving lectin-agglutinis with different specificities components exposed on the surfaces of pathogens (Crouch *et al.*, 2000; Chiou *et al.*, 2000; Kawabata *et al.*, 2001). As shown in Table 2, five types of lectins (tachylectins(TL)-1 to 5), three types of C-reactive proteins with different binding specificities to phospholipids, and several agglutinins have been identified in circulating hemocytes and hemolymph plasma (Kawabata and Iwanaga, 1999; Kawabata and Tsuda, 2002). These components function synergistically to form an effective host defense against invading microbes and foreign substances.

Of these agglutin-lectins, hemocyte-derived TL-1 interacts with Gram-negative bacteria probably via 2-keto-3-deoxyoctonate (KDO), a constituent of bacterial LPS, and was found to cause the agglutination of sheep erythrocytes coated with LPS (Saito *et al.*, 1995). TL-1 is a single-chain protein of 221 amino acid residues with no *N*-linked sugar chain, and contains three intra-chain disulfide bonds and a free cysteine residue. The six tandem repeats of TL-1 are an outstanding structural feature. TL-1 exists as a monomer in solution, and the 3D structure of TL-1 is dominated by six β sheets, which correspond to the six tandem repeats (Fig. 5). TL-1 has a six-bladed propeller structure with a central zinc atom coordinated with three aspartate residues, two serine residues and one molecule of water. The center of TL-1 also contains a cluster of six lysine residues one in each of the six repeated structures, which form a hole allowing interactions with trisaccharides containing KDO (for more details see, Saito *et al.*, 1995; Kawabata and Iwanaga, 1999).

TL-2 is a unique lectin and has hemagglutinating activity against human A-type erythrocytes (Okino *et al.*, 1995). TL-2 binds specifically to GlcNAc with a dissociation constant of 0.05 mM. A detailed sugar-binding analysis indicated that the acetamide group at the C-2 position and the free OH group at the C-4 position of GlcNAc are required for recognition by TL-2. The most interesting feature of the 236 amino acid TL-2 sequence is the five tandem 47 amino acid repeats. Moreover, TL-2 does not contain cysteine or *N*- or *O*-linked sugars, and is present as a monomer in solution. The X-ray structure of TL-2 complexed with GlcNAc has been elucidated at a resolution of

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2.0Å (Fig. 5). TL-2 has a five-bladed β-propeller structure and its single chain is organized into five sheets, arranged in consecutive order with five-fold rotational symmetry around a central tunnel (Beisel *et al.*, 1999). The structure contains five equivalent binding sites, with virtually identical occupancies and geometries in the crystal.

GlcNAc points its acetamide group toward the bottom of the TL-2 binding pocket, whereas the C6-OH group is directed out into the medium. Moreover, TL-2 shows virtually no change in main or side chain conformation after binding GlcNAc. The nature of its binding pocket explains its strict specificity toward GlcNAc. Thus, the presence of five binding sites per TL-2 molecule suggest that it recognizes pathogens with a fairly high ligand density of GlcNAc units. Moreover, the multiple binding of the five binding sites to repetitive structures on pathogens would generate strong interactions, indicative of a type of pattern recognition.

TL-3 exhibits hemagglutinating activity specifically against human A-type erythrocytes. The hemagglutinating activity of TL-3 is equivalent to that of TL-2, but its activity is not inhibited by *D*-GlcNAc or *D*-GalNAc (Saito *et al.*, 1997). Interestingly, its hemagglutinating activity is completely inhibited by a synthetic pentasaccharide derivative of blood group A antigen and more strongly inhibited by the S-type LPSs of several Gram-negative bacteria in the concentration range 5-10 ng/ml, but not by the corresponding R-type LPSs, which indicates that TL-3 has high specificity against O-antigens. Moreover, TL-3 contains 123 amino acid residues in the form of two repeating sequences and is present as a dimer in solution (Saito *et al.*, 1997).

TL-4 is a 470kDa 232 amino acid oligomeric glycoprotein (Inamori *et al.*, 1999), and its hemagglutinating activity against human A-type erythrocytes is more potent than those of TL-2 and TL-3. Although *L*-fucose and *N*-acetylneuraminic acid at 100 mM completely inhibits this activity, TL-4 is more strongly inhibited by bacterial S-type LPS, but is not inhibited by R-type LPS, which lacks O-antigen. Thus, colitose (3-deoxy-*L*-fucose), a unique sugar present in the O-antigen of *E. coli* O111: B4, and which is structurally similar to *L*-fucose, is a likely specific TL-4 ligand.

Of the five types of plasma-derived tachylectins, TL-5A and TL-5B have greatest hemolymph agglutinating activities, and exhibit broad specificity for substances containing *N*-acetyl groups (Gokudan *et al.*, 1999). TL-5A and TL-5B agglutinate all types of human erythrocytes, indicating that the primary recognition substance is not a blood group antigen. Their hemagglutinating activities are inhibited by 5 mM EDTA, and this inhibition is neutralized by adding an excess of $CaCl_2$. Both TL-5A and TL-5B specifically recognize acetyl group-containing substances including noncarbohydrates; only the acetyl group is required for recognition. They also strongly agglutinate Gram-negative and-positive bacteria. The overall sequence identity between the 269 amino acid residues of TL-5A and the 289 residues of TL-5B is 45%, and they share sequence similarity with the COOH-terminal fibrinogen-like globular domain. Interestingly, a collagenous domain in ficolins is missing in the corresponding regions of TLs-5A and -5B. Thus, since ficolin-like mannan-binding lectin is known to participate in a novel lectin pathway of the complement system (Matsushita and Fujita, 2001; Lindahl *et al.*, 2000), TLs-5A and -5B, both structural homologs of ficolin, may also trigger the activation of the horseshoe crab complement system (Fujita, 2002; Gadjeva *et al.*, 2001). The 3D structure of TL-5A complexed with GlcNAc has been recently elucidated using the multiple isomorphous replacement technique (Fig. 5). It is an ellipsoidal molecule with overall dimensions of ca. 34 X 36 X 53Å, and is subdivided into three distinct interacting domains (Kairies *et al.*, 2001). TL-5A contains a Ca^{2+}-binding site and an acetyl group-binding pocket. In addition, a systematic computer search performed with SCOP revealed that TL-5A is structurally related to the fibrinogen γ-chain fragment (rms deviation of 1.09Å, overall), thus verifying our previous prediction based on amino acid sequence similarity that TL-5A showed the highest sequence identity (53%) to that of mammalian ficolins (Gokudan *et al.*, 1999). Electron microscopy showed that TL-5A forms a three- or four-bladed propeller structure, and that TL-5B has a two-bladed propeller structure. If their ligand binding sites are located in their blades, TLs-5A and -5B could be more potent against high-density ligands on pathogens, and their polyvalent binding of acetyl-groups a key factor of microorganism binding. In addition they function as non-self-recognizing proteins.

The hemolymph of horseshoe crabs contains another class of bacterial agglutinins, which is structurally related to mammalian CRP (Iwaki *et al.*, 1999). CRP was first recognized in human plasma as a non-immunoglobulin capable of precipitating with C-polysaccharides derived from the cell wall of *Streptococcus pneunomiae*. Limulin, a sialic acid- and phosphorylethanolamine-binding hemagglutinin in the hemolymph plasma of *L. polyphemus*, is a hemolytic CRP (Table 2). Three types of CRPs have been purified from the plasma of *T. tridentatus*, by using their different affinities for fetuin-agarose and phosphorylethanolamine-agarose. These CRPs are named *T. tridentatus* CRP-1 (tCRP-1), tCRP-2, and tCRP-3, each of which consists of several isoproteins. tCRP-2 and tCRP-3, but not tCRP-1, agglutinate mammalian erythrocytes. tCRP-1 is the most abundant CRP, and exhibits highest affinity for phosphorylethanolamine-protein conjugate but lacks both sialic acid-binding and hemolytic activities. tCRP-1 binds to both fetuin-agarose and phosphorylethanolamine-agarose, and exhibits Ca^{2+}-dependent hemolytic and sialic acid-binding activities. Furthermore, tCRP-2 exhibits a higher affinity to colominic acid, a bacterial polysialic acid, though tCRP-3 shows stronger hemolytic, sialic acid-binding and hemagglutinating activities than tCRP-2. However, tCRP-3 has no affinity for phosphorylethanolamine-agarose or colominic acid. Thus, tCRP-3 is a novel hemolytic CRP, which lacks the common characteristic of CRPs, namely, the ability to bind phosphorylethanolamine-agarose. Twenty-two clones of tCRPs

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with different deduced amino acid sequences showing high levels of molecular diversity have been identified (Iwaki *et al.*, 1999). Moreover, of these tCRP clones only tCRP-3 contains a unique hydrophobic nonapeptide sequence that appears in the transmembrane domain of a major histocompatibility complex class I heavy chain of rainbow trout, which suggests that their hydrophobic nonapeptide patch is important for the hemolytic activity of tCRP-3. The structural and functional diversities of tCRPs offer an interesting model for the study of invertebrate innate immunity, which allows survival without the benefit of acquired immunity.

Antibacterial, antifungal, and antiviral systems The S-granules of *T. tridentatus* hemocytes contain a family of arthropodous peptide antibiotics, called the "tachyplesin" family, in addition to tachycitins, tachystatins, and β-defensin analogs (Table 2). Moreover, the L-granules of *T. tridentatus* were found to contain an antibacterial protein, called anti-LPS factor, factor D and big defensin (Fig. 3). The S-granule-derived peptides antibiotics bind chitin, but no other polysaccharides, e.g., cellulose, mannan, xylan, or laminarin. One of these antimicrobial peptides (Table 2) tachyplesin, which consists of 17 amino acid residues, was found to significantly inhibit the growth of Gram-negative and -positive bacteria and fungi (Iwanaga *et al.*, 1998; Morvan *et al.*, 1997). Tachyplesin has a rigid hairpin loop that is constrained by two disulfide bridges and adopts an antiparallel β-sheet conformation connected to a β-turn (Fig. 5). In its planar conformation, the five hydrophobic side chains are localized on one face, and the six cationic side chains on another. Thus, the amphiphilic structure of tachyplesin is presumed to be closely associated with its antibacterial activity.

Big defensin, which consists of 79 residues, is unlike mammalian defensins in terms of its size (though they have 29-34 residues in common), and is proteolytically divided into two domains by trypsin. Moreover, its COOH-terminal domain shows sequence similarity with mammalian neutrophil-derived β-defensins. In fact, the disulfide motif in this domain is identical to that of bovine neutrophil β-defensin. One noteworthy characteristic of *T. tridentatus* big defensin is that its two domains are functionally different. Big defensin has antimicrobial activity against both Gram-negative and -positive bacteria. Interestingly, its NH_2-terminal hydrophobic domain is more effective than its COOH-terminal defensin domain against Gram-positive bacteria, whereas its COOH-terminal domain more potent against Gram-negative bacteria, which suggests that big defensin is a chimeric defense molecule. Thus, big defensin in *T. tridentatus* may represent a new class of the defensin family, possessing two functional domains with different antimicrobial activities.

Tachystatins A, B, and C, consist of 41-44 residues, and exhibit broad-spectrum antimicrobial activity against fungi and Gram-negative and -positive bacteria (Osaki *et al.*, 1999).

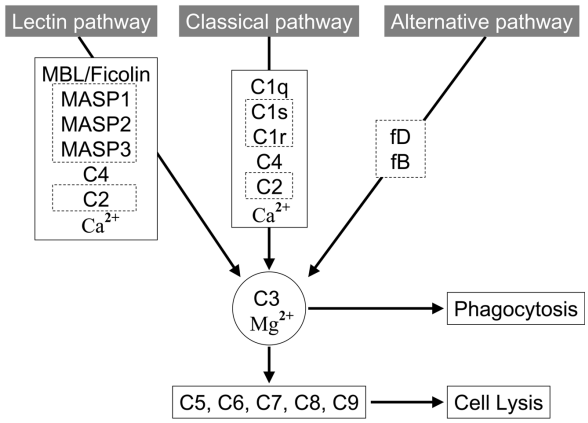

Fig. 6. Outlines of the classical, lectin, and alternative pathways of complement activation. The complements boxed and scored are serine proteinases. MBL, mannose binding lectin; MASP, mannose-binding lectin-associated serine protease.

Of these three, tachystatin C is the most effective, and tachystatin A is homologous to tachystatin B. Tachystatin C contains the same disulfide motif found in tachystatin A, but shares low sequence similarity with tachystatin A, which shares sequence similarity with ω-agatoxin-IVA of funnel web spider venom, a potent blocker of voltage-dependent calcium channels. However, tachystatin A does not block P-type calcium channel activity in rat Purkinje cells. Tachystatins cause morphologic changes in budding yeast, and tachystatin C has strong cell lysis activity. Furthermore, tachystatin C, but not A and B, has hemolytic activity, and shows sequence similarity with several arachnid neurotoxins. Thus, as horseshoe crabs are close relatives of spiders, tachystatins and spider neurotoxins may have evolved from a common ancestral peptide. Recently, the 3D structure of tachystatin A was determined by NMR (Fig. 5), and its 2D structure was found to contain a cysteine-stabilized triple-standard β-sheet domain which undergoes amphiphilic folding to form the 3D structure as is observed for many membrane-interactive peptides (Fujitani *et al.*, 2002). Interestingly, tachystatin A shares 3D structural similarities with ω-agatoxin IVA. These structural comparisons and functional correspondences suggest that the antifungal activities of both peptides mechanistically resemble those of defensins.

Tachycitin consists of 73 amino acid residues that contain five disulfide bonds each with no *N*-linked sugar (Kawabata *et al.*, 1996). Moreover, although its antimicrobial activity is only moderate, tachycitin synergistically enhances the antimicrobial activity of big defensin, for example the IC_{50} value of big defensin against Gram-negative bacteria reduces by a factor of 50 in the presence of small amounts of tachycitin. As shown in Fig. 5, the 3D structure of tachycitin is largely composed of NH_2- and COOH-terminal domains (Suetake *et al.*, 2000). In the latter, tachycitin forms a hairpin

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Table 3. Major defense molecules found in hemocytes, hemolymph plasma and tissues of the fresh water crayfish (*Pacifastacus leniusculus*)

Proteins and peptides	Mass(kDa)	Function
Prophenoloxidase system		
Prophenoloxidase(ProPO)	76	Precursor of phenoloxidase and melanin formation. Serine protease
ProPO activating enzyme (ppA)	48	which contains β-defensin-like domain.
LPS and β 1,3-glucan binding proteins (LGBP)		
β 1,3-glucan binding protein	100	One of the triggers of prophenoloxidase activating system (ProPO system).
LGBP	40	Involvement in ProPO system. Opsonin and cell adhesion.
Mannose-binding lectin	28	secreted from hemocytes upon challenge.
Cell adhesion proteins		
Peroxnectin	76	Cell adhesion molecule and, binding to peroxidase.
Masquerade-like protein 1	124/134	Cell adhesion protein which contains non-catalytic serine protease domain.
Masquerade-like protein 2	40	secreted from hemocytes upon challenge.
Proteinase inhibitor		
α_2-Macroglobulin	190 X 2	Complement-like activity.
Pacifastin	155	Serine protease inhibitor which contains a unique transferring chain.
Subtilisin inhibitor	28	
Kazal-type inhibitors	10-30	Four domains-Kazal type protease inhibitor.
Antibacterial proteins		
Lysozyme	14	Cell lysis.
Hemagglutinin	420	Hemaggultinating activity.
Cytosolic ferritin	440	A storage protein for ferric ion.
Astocidine 1	1.6	Antimicrobial peptide.
Astocidine 2	1.8	Antimicrobial peptide.
Curcinine-like peptides	14-20	Antimicrobial peptide.
Anti-LPS factor	13.5	secreted from hemocytes upon challenge.
Others		
Vitellogenin-related protein	210 X 2	A plasma clotting protein, lipoprotein-like, and TGase substrate.
Transglutaminase (TGase)	90	Cross-linking.
Hemocyanins	360	O_2 transporter, phenoloxidase-like activity, and produces anti-microbial peptides.
Astakine	8.7	Cytokine-like activity in hematopoiesis.

loop of a two-stranded β-sheet, which superimposes the structure of the chitin-binding site of hevein without any sequence similarity. Hevein is an antifungal peptide that was isolated from the rubber tree *Havea basiliensis*. Tachyplesin like tachycitin also has strong chitin-binding ability, and contains a similar hairpin loop in a two-stranded β-sheet (Fig. 5). In both tachycitin and tachyplesin hydrophobic residues clustered on the one face of the β-hairpin loops probably function as the chitin-binding site. Chitin is a component of the cell wall of fungi, and is also a major structural component of arthropod exoskeletons. Thus, the antimicrobial substances released from *T. tridentatus* hemocytes probably recognize chitin exposed at the site of a lesion, and appear to function in wound healing and as antibacterial molecules, in addition, they may stimulate and accelerate biosynthesis of chitin at sites of injury.

The freshwater crayfish *(Pacifastacus (P) leniusculus)*

Much progress has been achieved on the innate immune system of fresh water crayfish, *Pacifastacus leniusculus* (Fig. 2B) by Söderhäll and his colleagues (Söderhäll *et al*., 1996). Here, we focus on the prophenoloxidase activating system (the pro-PO system) and the non-self recognition system, both of which have important roles in host defense.

The pro-PO activating system Table 3 summarizes the various defense proteins and peptides found in crayfish hemolymph and tissues. The crayfish pro-PO system consists of

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several proteins that participate in melanin formation, cytotoxic reactions, cell adhesion, encapsulation, and phagocytosis. A similar immune system is found in many other invertebrates, such as, insects, ascidians, mollusks, echinoderms, millipedes, bivalves, and brachiopods (Söderhäll *et al.*, 1994; Cannon *et al.*, 2004). It is an efficient non-self recognition system and is initiated by the recognition of the LPSs or peptidoglycans of bacteria and (1 → 3)-β-D-glucans of fungi. The pro-PO system of *P. leniusculus*, which is similar to those of insects described later, is composed of a protease cascade composed of a pattern of recognition proteins (LPS and (1 → 3)-β-D-glucan binding proteins), several serine protease, their zymogens, and pro-PO (Table 3). The active form of pro-PO, phenoloxidase, also known as tyrosinase, catalyzes two successive reactions; the first is the hydroxylation of a monophenol to an O-diphenol, and the second is the oxidation of O-diphenol to O-quinone (Sugumaran, 2002). The production of toxic quinone intermediates and O-quinones by phenoloxidase is an initial step in the biochemical cascade of melanin biosynthesis, and is also important in cuticular scleotization, wound healing, and in the encapsulation of foreign materials for host defense (Cerenius and Söderhäll, 2002). During the last five years, structural information about pro-PO activating enzymes (serine protease) have become available. In crayfish, pro-PO is synthesized and localized in hemocyte granules and then released into plasma by exocytosis triggered by pattern recognition proteins (Table 3). The so called (1 → 3)-β-D-glucan/LPS binding proteins (Cerenius *et al.*, 1994), are the triggering molecules of the crayfish pro-PO system, since they bind microbial cell wall components and induce the activation of serine protease zymogens in the pro-PO system (Lee *et al.*, 2000). The serine proteases of the crayfish pro-PO system contain one "clip domain", which shows homology with horseshoe crab-derived big defensin and mammalian β-defensin mentioned before. This "clip domain" is cleaved upon activation by upstream proteases (Wang *et al.*, 2001). Moreover, the cleaved "clip domain" in crayfish shows antibacterial activity *in vivo* against Gram-negative bacteria, which suggests that the pro-PO activating serine proteases have a dual function.

The pro-PO system is controlled carefully by regulating proenzyme activation and phenoloxidase activity, in which it produces highly toxic quinone intermediates (Johansson *et al.*, 1995). This control is achieved partially by inactive zymogen, which is activated by proteolysis (Wang *et al.*, 2001). Zymogen is stored in circulating hemocytes in the crayfish, and to avoid excessive or premature activation, protease inhibitors also play an important role, as they do in the regulation of the *T. tridentatus* clotting cascade (Fig. 4). In the case of the freshwater crayfish detailed biochemical data is available on the manner in which such inhibitors regulate the activation of the pro-PO system. One of the most efficient serine protease inhibitors is a 155 kDa heterodimeric protein with a unique structure, named pacifastin (Liang *et al.*, 1997). It is composed of one heavy chain with transferrin lobes and one protease inhibitor-light chain, which are covalently linked to each other. Interestingly, the pacifastin light chain shares a characteristic cysteine array with a family of monomeric low molecular weight protease inhibitors of the serpin type; moreover, this cysteine array has not been found in other protease inhibitors. As the crayfish-derived inhibitor was the first member of this family isolated, these inhibitors are now commonly referred to as members of the pacifastin family of protease inhibitors. Other inhibitors also present in crayfish hemolymph plasma have some capacity to restrict serine protease activity, such as α_2-macroglobulin and Kazal inhibitor (Johansson *et al.*, 1994) (Table 3).

Pattern-recognition molecules As described previously, the presence of minute quantities of microbial surface components, such as (1 → 3)-β-D-glucan or peptidoglycan, are enough to trigger the activation of the pro-PO system. A large number of pattern-recognition molecules with affinities for bacterial and fungal cell wall components that mediate pro-PO system activation have been identified. In freshwater crayfish, two separate pattern-recognition proteins, namely, (1 → 3)-β-D-glucan binding protein and masquerade-like protein are known (Huang *et al.*, 2000), and their structure-function relationships have been studied in detail (Cerenius *et al.*, 2003). Of these, the latest to be identified has structural properties that are similar to those of *Drosophila*-derived masquerade; a serine protease homologue expressed during embryogenesis, laval, and pupal development in *D. melanogaster* (Lee and Söderhäll, 2001). The freshwater crayfish masquerade-like protein binds to formaldehyde (HCHO)-treated Gram-negative bacteria and HCHO-treated yeast (*Saccharomyces cerevisiae*), but not to HCHO-treated Gram-positive bacteria. Intact masquerade-like protein is present in crayfish hemocytes as a heterodimer of 134 kDa and 129 kDa subunits (Table 3). After binding to bacterial or yeast cell walls, this protein is processed by a proteolytic enzyme. The 134 kDa subunit yields four fragments of 64, 47, 33 and 29 kDa, and the 129 kDa subunit results in four fragments of 63, 47, 33 and 29 kDa. Of these fragments, the 33 kDa fragment derived from the COOH-terminal part of masquerade-like protein has cell adhesion activity. *E. coli* coated with masquerade-like protein are more rapidly cleared in crayfish than *E. coli* alone, which suggests that masquerade-like protein is an opsonin-like protein.

Proteins with components similar to those of *Drosophila* and freshwater crayfish masquerade proteins, with serine protease-inactive homologues, have been identified in vertebrates and invertebrates, and have been suggested to have various biological activities, e.g., antimicrobial activity (human azurocidin, horseshoe crab factor D (Table 1) (Kawabata *et al.*, 1996)), growth factor activity (hepatocyte growth factor), adhesion promoting activity (fruit fly glutactin, neurotactin, and masquerade), and immune activity (mosquito infection-responsive serine protease-like protein). These molecules show homology to serine proteases, except for catalytic residue substitutions. Therefore, the cell adhesion and opsonic activities of freshwater crayfish masquerade-like protein suggest that it plays a significant role in the innate immune system of this animal.

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Table 4. Major defense molecules and complements and its genes identified in an ascidian

(A) Various defense molecules (*Halocynthia roretzi*)
Phenoloxidase (62kDa) released from hemocytes.
Membrane glycoprotein (160kDa), named A74 protein, one of the adhesion molecules.
Galactose-specific lectin (600kDa).
N-Acetylglucosamine-specific lectin (50kDa).
N-Acetylgalactosamine-specific lectin (500kDa).
Trypsin inhibitors (8kDa).
LPS-binding hemagglutinin (120kDa).
Antimicrobial substances-Helocyamins
(0.6kDa, 3,4 dihydroxyphenylalanine (DOPA) containing peptides)
(B) Complements and its genes (*Halocynthia roretzi* and *Ciona intestinalis*)
Collectin/Ficolin
MASP/C1s/C1r
C3/C4/C5/α_2-Macroglobulin
Bf/C2
C6/C7/C8/C9/Perforin
Other defense proteins and structural domains predicted from genomic analysis (*Ciona intestinalis*)
Pentaxin, Cytokines (TNF), Toll-like receptors (three receptors), MyD88, IRAK, TRAF, IP105, NFκB, IκB, Immunoglobulin superfamily molecules (VCBP, Peroxidasin, Nectin, JAM/CTX, CD166), Fibrinogen-C, Sushi (SCR), C-type lectin, CUB, Integrin β, EGF, CD36, C1q, CD94.

The abbreviations: TNF, tumor necrosis factor; IRAK, IL-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; MASP, mannose-binding lectin-associated serine protease; SCR, short consensus repeat; CUB, C1r/Uegf/Bone morphogenetic protein 1.

Freshwater crayfish plasma clotting protein As described earlier, vertebrates and invertebrates have separately evolved efficient molecular mechanisms to immediately form blood clots (Iwanaga, 1993, Theopold *et al.*, 2004), which is essential to prevent blood/hemolymph loss in case of injury. Vertebrates have similar clotting systems, which result in the proteolytically induced aggregation of fibrinogen into insoluble fibrin. These noncovalently-associated fibrin aggregates are further stabilized by the formation of intermolecular covalent cross-links in the presence of factor XIIIa (transglutaminase) and Ca^{2+}. Ca^{2+}-dependent factor XIIIa catalyses covalent cross-linking to form an ε(γ-glutamyl) lysine between the side chains of specific lysine and glutamine residues on certain proteins (Tokunaga and Iwanaga, 1993). In the crayfish, hemolymph clotting is based on the direct transglutaminase-mediated cross-linking of a specific plasma protein, which is homologous to the vitellogenins (Hall *et al.*, 1995). Freshwater crayfish clotting protein is a lipoprotein that consists of 1,721 amino acids (210 kDa × 2, Table 3) and shares limited sequence similarity with other lipoproteins, such as mammalian apolipoprotein B and microsomal triglyceride transfer protein. It also contains a stretch with similarity to the D domain of von Willebrand factor (Hall *et al.*, 1999). Each of its 210 kDa subunits has lysine and glutamine side chains, which are covalently cross-linked to each other by transglutaminase. Hemolymph clotting is induced when a transglutaminase is released from hemocytes or tissues, and starts cross-linking plasma-derived clotting protein in the presence of Ca^{2+}. Moreover, crayfish clotting protein exists in both sexes, unlike the female-specific vitellogenins. In the presence of an endogenous transglutaminase, clotting protein molecules rapidly assemble into long and flexible chains that occasionally branch, as evidenced by electron microscopy. Despite its similar functions, clotting protein appears not to share any structural similarities with mammalian fibrinogen or horseshoe crab coagulogen. This indicates that crayfish clotting protein and lobster fibrinogen, which share sequence similarity with the vitellogenins, constitute a separate group of clotting factors. Thus, the crustacean clotting proteins are a second type of gel-forming proteins and are evolutionary related to vitellogenins, but they should not be considered as true vitellogenins, since they have completely different functions and are constitutively expressed in both sexes.

The solitary ascidian *(Halocynthia (H) roretzi)*

Ascidians are intriguing invertebrates from the viewpoint of the evolution of the innate immune system (Azumi *et al.*, 2003), not least because they lack acquired immunity. The ascidians belong to the protochordata, and occupy a phylogenetic position between vertebrates and invertebrates. Of the ascidians, the largest in size is the solitary ascidian, *H. roretzi*, which inhabits the seas around Japan (Fig. 2C); large quantities of hemolymph and numbers of hemocytes can be obtained from the cavity of this animal. Biochemical studies on the cellular defense mechanisms used by ascidians have been mainly performed by Azumi and Yokosawa group. In *H. roretzi*, hemocytes show several cellular reactions, such as,

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hemocyte aggregation, phagocytosis, self and non-self recognition reactions, and phenoloxidase enzyme release in response to stimuli (Takahashi *et al.*, 1995). Table 4A shows major hemolymph-derived components that are involved in *H. roretzi* host defense. Of these components, phenoloxidase is contained in hemocytes and is released from hemocytes by treatment with zymosan or LPS, but not by (1 → 3)-β-D-glucan (Hata *et. al*., 1998). Like other phenoloxidases derived from crayfish and insects, *H. roretzi* phenoloxidase is a metalloenzyme that requires copper ions for full activity (Hata *et al.*, 1998). Interestingly, the phenoloxidase shows antibacterial activity against *E. coli* in the presence of *L*-(3,4-dihydroxy) phenylalanine (DOPA), which suggests that phenoloxidase catalyzes the formation of antibacterial substances from DOPA (see Table 4 (A)). Thus, hemocyte-derived phenoloxidase functions as a humoral factor in the host defense of *H. roretzi.*

On the other hand, as shown by Table 4(B), a number of possible complement genes have recently been identified in the ascidian *Ciona itestinalis* (Azumi *et al.*, 2003). In higher vertebrates, about 35 proteins are known to function in the three complement activations of the classical, alternative, and lectin pathways (Fig. 6) (Fujita, 2002, Caroll, 2004). Thus, it was surprising to learn that almost the same number of complement components are present in ascidians (Nonaka, 2001). In support of these findings, biochemical evidence now demonstrates the presence of mammalian-like alternative and lectin pathways in tunicates (Smith *et al.*, 1999), and in sea urchins (Smith, 1999, Kenjo *et al.*, 2001). In *H. roretzi* cDNA clones of third complement (C3), mannose-binding lectin associated serine protease (MASP), and factor B(Bf) were isolated from ascidian hepatopancreas cDNA library (Nonaka and Azumi, 1999). The deduced primary structure of ascidian complement C3 shows an overall similarity with mammalian C3, and contains a typical thioester site. Moreover, two distinct ascidian MASPs, namely, AsMASPa and AsMASPb, have the same domain structures as mammalian C1r/C1s/MASP-1/MASP-2 (Nonaka *et al.*, 1999), and both of C1r, C1s, MASP-1, and MASP-2 them are more similar to mammalian MASP-1 than to mammalian C1r/C1s/MASP-2 (Xin *et al.*, 1997). Ascidian body fluid contains opsonic activity that enhances the phagocytosis of yeast by ascidian hemocytes, which indicates that the lectin-dependent opsonic complement system, and the alternative-like pathway existed prior to the emergence of the vertebrates and well before the establishment of acquired immunity (Nonaka and Azumi, 1999). The presence of C1q in *Ciona* was unexpected, since mammalian C1q binds to immunoglobulins (Dodds 2002). However, human C1q also interacts with C-reactive protein (CRP), an acute phase proteins, and is a member of the pentaxin family, which is also found in horseshoe crab hemolymph plasma (Iwaki *et al.*, 1999). Since *Ciona* possess pentaxins (Table 4B), a pentaxin may have been the original partner of C1q. Interestingly, both C1q and pentaxin occur only in a deuterostome or chordate lineage (Fujita, 2002). In addition to the above complement components, many other genes involved in the innate immune system of the invertebrate chordate, *Ciona intestinalis*, have been identified, including three Toll-like receptors and the genes involved in the intracellular signal transduction of immuno responses, such as MyD88, IL-1 receptor-associated kinase (IRAK), TNF receptor-associated factor (TRAF), NFκB, and IκB. The *Ciona* genome also contains three IL-1 receptor-like genes (IL-1R), one tumor necrosis factor gene (TNF), and three TNF-receptor-like genes (Azumi *et al.*, 2003). With respect to protein molecules involved in phagocytosis and pathogen recognition, the *Ciona* genome contains several α and β integrins, C-type lectins, scavenger receptor-like protein, Gram-negative bacteria binding protein, and LPS-binding protein (Table 4B). However, no report has been issued on the ascidian hemolymph clotting system.

On the other hand, it is known that *H. roretzi* hemolymph contains several lectins, including a galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine-specific lectins, and LPS-binding hemagglutinin, all of which are involved in internal defense mechanisms (Table 4A). Purified galactose-specific lectin, which is present in large amounts in *H. roretzi* hemolymph plasma, strongly enhances the phagocytosis of sheep erythrocytes by *H. roretzi* hemocytes, which suggests that it is a candidate opsonin. Moreover, it is interesting that galactose-specific lectin induces active oxygen (O_2^-) production by mammalian leucocytes (Azumi *et al.*, 2000). *H. roretzi* hemocytes produce O_2^- in response to treatment with zymosan, thus, indicating that galactose-specific lectin regulates several cellular reactions in the host defense system (Vasta *et al.*, 1999). Moreover, a search and analysis of immunity-related genes in *Ciona* suggested the presence of a well and uniquely developed innate immune system in urochordates. In particular, the urochordata genome is expected to provide information on the ancestral state prior to the two rounds of complete genome duplication that are known to have occurred at the early stage of vertebrate evolution (Ohno, 1994). In addition to the horseshoe crabs, freshwater crayfish and ascidians mentioned above, a number of defense molecules that include antimicrobial substances, lectins, (1 → 3)-β-D-glucan, and LPS-binding proteins, Toll-like receptors, cell adhesion proteins, complement-like thioester containing proteins, and proteinase inhibitors have been isolated from many other invertebrates and characterized biochemically (Inamori *et al.*, 2000; Inamori *et al.*, 2004). As it is not our intention to introduce these molecules here, instead we cite the following reviews: Earthworm (Cooper *et al.*, 2003), Sea urchins (Smith, 1999), the Echinoderm *Cucumaria frondosa*, (Beauegard, *et al.*, 2001), Crustaceans (Smith and Chisholm, 2001), and Molluscs (Loker and Bayne, 1999).

The immune responses of insects

In the case of insects, the first-line defense barriers are; the outer exoskeleton, the peritrophic matrix of the midgut

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Table 5. Defense molecules found in insects

Protein and peptide	Mass (kDa)	Function
(A) *Drosophila melanogaster*		
Necortic	48	serine proteinase inhibitor
Persephone	40	serine proteinase
Spatzle	27	cytokine-like function
PGRP-SA	20	pattern recognition protein
GNBP	49	pattern recognition protein
Toll	110	leucine-rich repeat (LRR) protein
Myd-88	54	adaptor protein
Tube	46	adaptor protein
Cactus	50	ankyrin repeats mediate protein
Dorsal	67	transcription factor
Dif	67	transcription factor
PGRP-LC	50	d-pathway receptor (PGRP domain)
IMD	67	Zn-finger domain containing protein
Fadd	48	caspase8-like apoptosis regulator
TAK1	68	serine/threonine protein kinases
IRD5	75	serine/threonine protein kinases
DREDD	31	death related ced-3 like protein
Relish	97	transcription factor
(B) *Sarcophaga peregrina*		
Sarcotoxin I	3.5	antibacterial peptide
Sarcotoxin II	24	antibacterial peptide
Sarcotoxin III	7	antibacterial peptide
Sapecin	4	antibacterial peptide
5-S-GAD	0.573	antibacterial molecule
AFP	7	antifungal peptide
Sarcophaga lectin	32/30	galactose-binding lectin
Granuloctin	20	mucin-binding lectin
26/29 kDa protease	26/29	cathepsin-like protease

PGRP-SA, peptidoglycan recognition protein-short form A; GNBP-1, Gram-negative binding protein-1; Myd88, myeloid differentiation primary-response protein 88; Dif, dorsal-related immunity factor; PGRP-LC, peptidoglycan recognition protein-long form C; IMD, immune deficiency; Fadd, Fas-associated death domain; Tak1; transforming-growth-factor-β-activated kinase1; IRD5, immune response E deficient 5; RIP, receptor-interacting protein; DREDD, death-related ced-3/Nedd2-like protein; AFP, antifungal protein; 5-S-GAD, *N*-β-alanyl-5-*S*-glutathionyl-dopa.

epithelium, and the chitinous lining of the trachea. But, these physical barriers cannot prevent penetrations by pathogenic microbes. Once microbes have entered the haemocoel, they are exposed to humoral and cellular responses (Leclerc and Reichhart, 2004; Waterfield *et al.*, 2004; Abraham and Jacobs-Lorena, 2004). Humoral defenses include the production of antimicrobial peptides, the induction of lectin synthesis, and the activation of the pro-PO system, whereas cellular defenses involve hemocyte-mediated immune responses, which include phagocytosis and encapsulation. The differentiation of humoral and cellular defenses is not distinct as many humoral factors affect hemocyte function. Studies regarding insect defense reactions in different insect models have identified several important defense molecules that sense invading microorganisms. Although most studies of humoral and cellular reactions have been performed in large-bodied insects such as *S. perigrina* (Fig. 2E), *B. mori* (Fig. 2F), *M. sexta* (Fig. 2G), *T. molitor* (Fig. 2H-1), and *H. diomphalia* larvae (Fig. 2H-2), excellent genetic and molecular level investigations have been performed in *D. melanogaster* (Fig. 2D). Table 5 summarizes the various defense molecules found in insects. Our knowledge of innate immunity in insects and mammals has increased dramatically (Hoffmann, 2003; Iwasaki and Medzhitov, 2004). The recruitment of a similar pattern of recognition receptors and pathways in insects and mammals to stave off infection suggests that they have developed similar mechanism and molecular pathways to recognize and eliminate pathogenic invaders. Figure 2 (D-H) shows insects that have been used as model systems by studies on insect innate immunity. Here we summarize the innate immune-related molecules in insects.

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Table 5. Continued

Protein and peptide	Mass (kDa)	Function
(C) *Bombyx mori* and *Manduca sexta*		
B. mori		
pro-PO	70	melanin synthesis
Bombyx PGRP	20	PGN recognition protein
Bombyx βGRP	50	β-1,3-glucan recognition protein
PPAE	40	pro-PO activating enzyme
M. sexta		
Immulectin-4	32	encapsulation and melanization enhancer
Immulectin-3	33	LPS-specific lectin
Manduca βGRP	50	β-1,3-glucan recognition protein
serpin	40	serine protease inhibitor
plasmocyte-spreading peptide	13	paralytic peptide
PPAE	40	pro-Po activating enzyme
pro-PO	70	melanin synthesis
(D) *Holotrichia diomphalia* and *Tenebrio molitor*		
Holotricin-1	40	antibacterial peptide
Tenecin-1	40	antibacterial peptide
Holotricin-2	65	antibacterial peptide
Tenecin 2	65	antibacterial peptide
Holotricin-3	75	antifungal peptide
Tenecin-3	75	antifungal peptide
Tenebrio βGRP	45	β-1,3-glucan recognition protein
Holotrichia PGRP-1	20	pattern recognition protein
Holotrichia PGRP-2	18	pattern recognition protein
75 kDa protein	75	encapsulation-related protein
PPAF-I	35	easter-type serine protease
PPAF-II	45	masquerade-type serine protease
PPAF-III	35	easter-type serine protease
Holotrichia pro-PO	70	melanin synthesis
Tenebrio pro-PO	70	melanin synthesis
Calreticulin	40	encapsulation protein

pro-PO, pro-phenoloxidase; PPAE, pro-PO activating enzyme; PPAF, pro-phenoloxidase activating factor; βGRP, β-1,3-glucan recognition protein.

Drosophila (D) melanogaster. Two important pattern recognition receptors, Toll and PGN recognition protein-LC (PGRP-LC), which sense microbial infection upstream of the Toll pathway (activated mainly by fungi and Gram-positive bacteria) and the immune deficiency (IMD) pathway (activated mainly by Gram-negative bacteria), respectively, have been found. Figure 7 shows two main signaling pathways that regulate the expressions of the antimicrobial genes of *Drosophila*, which have been extensively reviewed recently (Gobert *et al.*, 2003; Brennan *et al.*, 2004; Hultmark, 2003). Activation of the Toll receptor by its ligand spätzle leads to the formation of a multimeric receptor-adaptor complex, which comprises three death-domain proteins: MyD88, Tube, and the kinase Pelle. The assembly of these proteins into a complex induces phosphorylation of Ik-B-like inhibitor Cactus (by an unknown kinase distinct from Pelle). Phosphorylated cactus is degraded by proteasomes and dissociates from Rel transcription factor dorsal-related immunity-factor (DIF), which is then free to translocate into the nucleus and activate numerous antimicrobial genes of *Drosophila*. The IMD pathway is probably triggered by an interaction between the transmembrane receptor PGRP-LC and peptidoglycan from Gram-negative bacteria [diaminopimelate (DAP)-type peptidoglycan]. The activation of this pathway triggers a cascade of kinases (DmTAK), which ultimately phosphorylate the Rel protein Relish. Phosphorylated Relish is then cleaved by DREDD caspase to remove the Ik-B like domain from the Rel DNA binding domain. Ik-B can then translocate into the nucleus, where it

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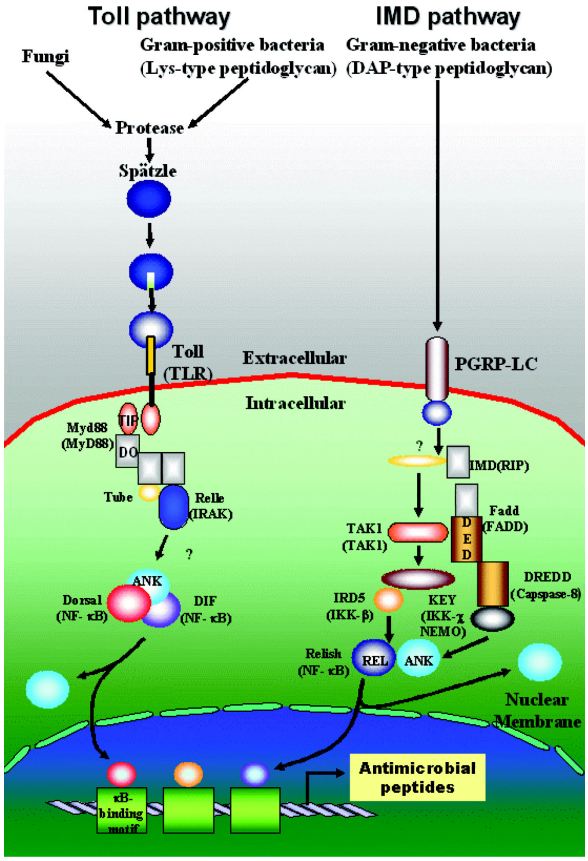

Fig. 7. The Toll and IMD pathways of *Drosophila* system.

regulates the transcription of immune related genes. These two pathways represent a typical paradigm of innate immune-response regulation in *Drosophila*, and demonstrate how two distinct signaling cascades can modulate the expression of a complex transcriptional programme in response to different pathogenic microbes.

Moreover, the antifungal peptide gene, Drosomycin, is predominantly activated by the Toll pathway in response to fungal and some Gram-positive bacteria infections. However, the antibacterial peptide gene, Dipertericin, is mainly activated by the IMD pathway in response to Gram-negative or some Gram-positive bacterial infections. Also, the Toll and IMD pathways share similarities with the interleukin-1 (IL-1/TLR and Tumor Necrosis Factor- (TNF-α) pathway that regulates NF-kB in mammals. The concept of the conservation of immune response in insects and mammals is now invigorating the field of innate immunity. In particular, the discovery of human Toll-like receptor (TLR) represented a turning point in the study of the mammalian immune system and opened many new research possibilities, which supports the notion that *Drosophila* presents a good model for the analysis of immune-response pathways.

The identification of PGRPs as pattern recognition receptors upstream of the Toll and IMD pathways, have raised the following pivotal questions. How can the bacterial sensing specificities of PGRPs be explained at the molecular level? and, How do PGRPs send signals downstream? Recently, the first crystal structure of *Drosophila* PGRP-LB was reported (Kim *et al.*, 2003). This structure demonstrates that this catalytic PGRP contains an active site cleft with a zinc cage, and that surface residues at the cleft are poorly conserved, thus indicating that PGRPs show widely varying individual specificities for molecular patterns on microbial cell walls. Behind this cleft, the presence of a distinctive hydrophobic groove suggested that the opposite face of PGRP subserves subsequent signaling after PGRP binding to the polymeric bacterial cell wall.

Sarcophaga (S) peregrina. Natori and his colleagues concentrated on this insect model and have isolated numerous antibacterial peptides, humoral lectins, and antifungal protein from fleshfly, *S. peregrina*, larvae. They studied the involvements of these proteins in self-defense and development, especially in metamorphosis (Natori *et al.*, 1999), and their findings suggest that immunity and development are deeply related. Recently, they isolated two novel antimicrobial molecules, named 5-***S***-GAD and KLKLLLLLKLK-NH_2 (Leem *et al.*, 1996; Nakajima *et al.*, 1997). The former has the ***N***-β-alanyl-5-***S***-glutathionyl-dopa structure (Fig. 8), and is produced in response to bacterial infection. Interestingly, this molecule has shown novel anti-tumor activity against a human breast cancer cell line (MDA-MB-4355), indicating that 5-*S*-GAD has a cytotoxicity associated with the generation of hydrogen peroxide ((H_2O_2) and superoxide (O_2^-). They suggested that 5-*S*-GAD inhibits tyrosine kinase (important for tumor cell growth), because src-kinase phosphorylation was impaired by 5-*S*-GAD *in vitro* (Hori *et al.*, 1997). A novel synthetic antibacterial peptide KLKLLLLLKLK-NH_2 derived from the *Sarcophaga* antibacterial peptide Sapecin, showed significant chemotherapeutic activity in methicillin-resistant *Staphylococcus aureus*-infected mice and an ability to produce superoxide [O_2^-] anion after human neutrophil activation (Cho *et al.*, 1999). These results provide us with the opportunity to develop new chemotherapeutic agents from insect defense molecules.

Bombyx (B) mori and Manduca (M) sexta. Insect pathologists have observed melanized capsules and nodules around hypae invaded by fungi and bacteria, and have suggested that the synthesis of melanin pigment is triggered by fungi or bacteria. Several groups have shown that melanin is synthesized by phenoloxidase (PO), and that PO is present as an inactive precursor, pro-PO, in hemolymph. Also, in addition to their vital role in innate immunity, the POs of insects are important for the pigmentation and sclerotization of many insect tissues (Sugumaran, 2002; Tzou *et al.*, 2002). It was suggested that the activation of pro-PO is caused by the pro-PO activating

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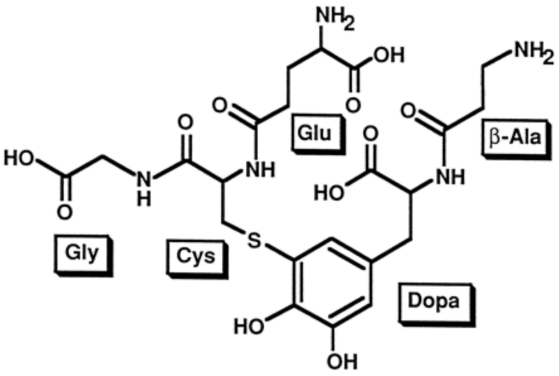

Fig. 8. The structure of 5-*S*-GAD.

system (or the pro-PO system) that involves; pattern recognition proteins capable of binding to pathogenic patterns, proteases that become active in the presence of microbial patterns; and other factors with the abilities to regulate the system, e.g. protease inhibitors. However, the elucidation of the activation mechanism of the insect pro-PO system is hampered by the fact that pro-PO is spontaneously activated when hemolymph is collected via an integumental incision. *B. mori* (silkworm) and *M. sexta* (tobacco hornworm) have proven to be good model systems for studying pro-PO activation system, because they may be obtained in large amounts of the hemolymphs from these insects.

Several important innate-immune-related proteins of the *Bombyx* pro-PO system have been purified and characterized by Ashida and colleagues. In particular, silkworm PGRP and β-1,3-glucan binding protein (bGRP) were first purified from hemolymph and identified as peptidoglycan and β-1,3-glucan pattern recognition proteins, respectively (Yoshida *et al.*, 1996; Ochiai and Ashida, 1988). These proteins are also known to induce the activation of the pro-PO system *in vitro*. *Bombyx* PGRP is a 19 kDa protein and was named because of its selective affinity for Gram-positive bacterial peptidoglycan. It was demonstrated that the binding of purified PGRP required a peptidoglycan with a glycan portion larger than at least two or more repeating units. In addition, PGRP bound peptidoglycan had no detectable amidase activity. The study of PGRP led to the cloning of PGRP orthologues from other animals, and these studies have revealed that PGRPs form an evolutionarily conserved family of proteins. βGRP was identified in the hemolymph of immuno-challenged silkworms and was found to have a structure similar to that of Gram-negative bacteria binding protein (GNBP) (Lee *et al.*, 1996). Moreover, βGRP/GNBP homologues have been isolated from multiple insect species (Fabrick *et al.*, 2004; Ma and Kanost, 2000; Zhang *et al.*, 2003), but are absent from the vertebrate genome, though one orthologue was found in the sea urchin (Bachman and McClay, 1996). The COOH-terminal domain of GNBP/βGRP shares sequence similarity with bacterial β-1,3-glucanase, and substitutions of the amino acid residues required for its catalytic activity prevented the activation of both of these entities, as is the case for PGRP bound to peptidoglycan. However, it remains unclear how PGRP and bGRP exert their biological effects and activate the next component of the pro-PO cascade when they bind to microbial triggers.

Regarding the pro-PO system, the Ashida group cloned *Bombyx* pro-PO, which shows high homology with the copper-binding site sequences of arthropod hemocyanins, and identified the pro-PO cleavage sites targeted by pro-PO activating enzyme (PPAE) (Kawabata *et al.*, 1995). They proposed that the activation mechanism of *Bombyx* pro-PO system involves PGRP, bGRP, pro-BAEEASE (an uncharacterized enzyme capable of hydrolyzing *N*-α-benzoylarginine-ethyl ester), pro-PPAE (*Drosophila* easter type-serine protease) and two pro-POs. However, the relationships between these factors and pro-POs remain unknown at the molecular level (Ashida and Brey, 1998).

The Kanost group cloned immulectin (32 kDa), a C-type lectin, from an *M. sexta* larval fat body cDNA library. Protein synthesis of immulectin was induced by injecting killed yeast or Gram-positive or Gram-negative bacteria, and its mRNA was induced in fat bodies by injecting bacteria (Yu *et al.*, 1999; Kanost *et al.*, 2004). Immulectin contains two carbohydrate recognition domains (CRDs). The COOH-terminal CRD most resembles that of LPS binding protein (Jomori and Natori, 1991) from the American cockroach (*Periplaneta americana*) and shares 26-35% identity with the CRDs of various mammalian C-type lectins (Ezekowitz *et al.*, 1988). Recombinant immulectin agglutinated yeast and Gram-negative bacteria. Interestingly, a combination of immulectin and *E. coli* LPS activated PO more rapidly than immulectin alone, whereas LPS alone had little effect on PO activation, indicating that immulectin synthesized in response to bacterial or fungal infections may help trigger a protective response in *M. sexta* in a manner resembling that of mannose-binding lectin, a C-type lectin that functions in the mammalian innate immune system. Also, the Kanost group published details of the characterization and functional analysis of 12 naturally occurring reactive site serpin variants from *M. sexta*. They found that 12 serpin variants were produced from a single gene during an analysis of more than 50 serpin cDNAs of *M. sexta* (Kanost, 1999). The NH_2-terminal 336 residues of these serpins were identical and they differed only at COOH-terminal 39-46 residues. One of the serpin variants, serpin-1J, strongly inhibited the activation of *M. sexta* PO, suggesting that serpin-1J may be an important regulator of endogenous serine protease(s), which are involved components of insect defense response to microbial infection (Jiang and Kanost, 1997).

Holotrichia (H) diomphalia and Tenebrio (T) molitor. One of the authors of the present review studied the antimicrobial proteins of two coleopteran insects. To date six groups of proteins have been purified and characterized, namely,

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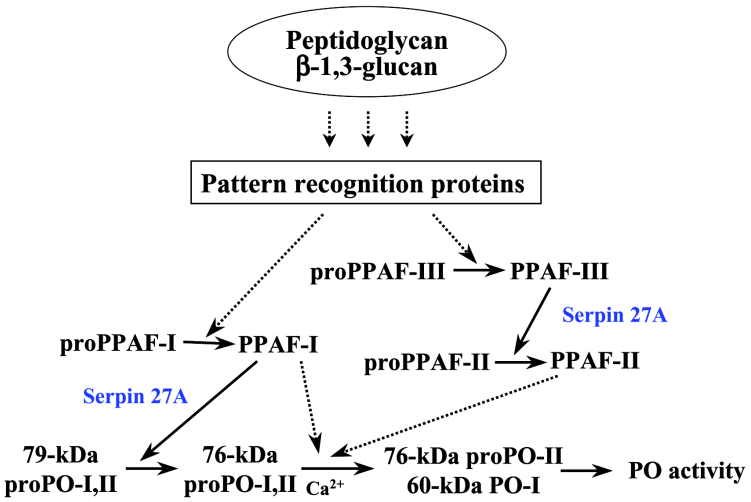

Fig. 9. Downstream part of pro-PO activation in *H. diomphalia* larvae.

Holotricin 1, 2, and 3, and Tenecin 1, 2, and 3. The antibacterial proteins, Holotricin 1 and 2, and Tenecin 1 and 2 are not normally present, but are promptly synthesized by the fat bodies and hemocytes and secreted into hemolymph when insects are in the acute phase of infection. Holotricin 1 and Tenecin 1 are a group of defensin or sapecin type antibacterial proteins and are more effective against Gram-positive bacteria. These proteins consist of 43 amino acids, which include six cysteine residues that form three intramolecular disulfide linkages (Moon *et al.*, 1994; Lee *et al.*, 1995a). Holotricin 2 and Tenecin 2 are beetle-specific antibacterial proteins that show potent bactericidal activity against Gram-negative bacteria. These proteins consist of 72 amino acid residues with no cysteine residues (Lee *et al.*, 1994). Holotricin 3 and Tenecin 3 are novel Gly- and His-rich proteins that consist of 84 amino acid residues and show significant activity against *Candida albicans* (Lee *et al.*, 1995b; Lee *et al.*, 1996). These proteins have no effects on the viabilities of Gram-positive or Gram-negative bacteria, but ionic condition seems to influence the antifungal activity of Tenecin 3. The obvious difference between Holotricin 3 and Holotricin 1 or 2 is that Holotricin 3 is a constitutively expressed protein of hemolymph, whereas Holotricin 1 and 2 are inducible.

The insect pro-PO system consists of two parts. The first of which is the recognition reaction between invading pathogens and pattern recognition proteins, and the second a signal triggered by pathogen invasion to down-stream pro-PO activating factor(s). The nature of the linkage between the up- and down-stream portions of the pro-PO system is unknown at the molecular level. Recently, we characterized three pro-PO activating factors (PPAF-I, -II and III) and two pro-POs (pro-PO-I and II), which are necessary for the activation of coleopteran *H. diomphalia* larvae. The overall structure of the 37 kDa pro-PPAF-I is almost identical to that of *Drosophila* easter, a serine protease essential for pattern formation during embryonic development. The mass of pro-PPAF-II (45 kDa) is similar to that of *Drosophila* masquerade, a non-proteolytic serine protease-like protein with a mutation in the catalytic triad (Lee *et al.*, 1998; Kwon *et al.*, 2000; Kim *et al.*, 2002). In addition, the structure of pro-PPAF-III is similar to that of easter serine protease, which can cleave 55 kDa pro-PPAF-II to generate 45 kDa PPAF-II. Furthermore, pro-PO-I and pro-PO-II (both 79-kDa) have been characterized in *Holotrichia*, and their structural changes during activation have been examined using *in vitro* reconstitution experiments. When these pro-POs were incubated with PPAF-I, both were converted to 76-kDa pro-POs, which did not exhibit any PO activity. However, when pro-PO-I or pro-PO-II were incubated with PPAF-I, pro-PPAF-II, or PPAF-III in the presence of Ca^{2+}, new bands of a 60-kDa PO-I (with PO activity) and of a 76-kDa pro-PO-II were detected. These results indicate that the conversions of *Holotrichia* pro-POs to their active forms with PO activity are accomplished by PPAF-I, PPAF-II, and PPAF-III through a two-step limited proteolysis in the presence of Ca^{2+}. As shown in Fig. 9, these detailed studies provided data for the first time on the relationship between two different pro-POs and three pro-PO activating factors in a large insect. Also, two pattern recognition proteins, *Holotrichia* PGRP and *Tenebrio* β-1,3-glucan-recognition protein, have been identified in two coleopteran insects (Zhang *et al.*, 2003; Lee *et al.*, 2004). Both proteins are involved in the activation of β-1,3-glucan dependent pro-PO activation. Interestingly, these pattern recognition proteins were specifically degraded following the activation of pro-PO with β-1,3-glucan and calcium ions. We proposed that this degradation pattern of recognition proteins is an important regulator of insect pro-PO system activation. However, it remains to be determined how the connection between the upstream and downstream portions of the pro-PO

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cascade is organized and activated at the molecular level.

Conclusion

During the last two decades, much progress has been made in the understanding of the invertebrate innate immune system. These studies at the cellular, biochemical, and genetic levels have led to discoveries of novel defense reactions and protein molecules, and also of new effector pathway mechanisms elicited after the recognition of "nonself". These results have provided new insights of the roles of host defense molecules and have resulted in the adoption of new invertebrate immunology concepts. This review summarizes the present status of invertebrate innate immune systems, by focusing on the molecular structures and functions of various defense components identified in invertebrate hemolymph and tissues. The data collected here indicate that individual species have characteristic patterns of host defense that depend on a wide variety of biological responses. Even in Arthropods, large differences in host defense systems are apparent between insects, the horseshoe crab, and the freshwater crayfish. For instance, insects and crayfish utilize a strong prophenoloxidase activating system as a biosensor that can react with various epitopes, such as LPS, peptidoglycan, and $(1 \rightarrow 3)$-β-D-glucan. In contrast, the prophenoloxidase activating system of the horseshoe crab appears to be a less important component of the host defense, but provides a strong LPS /$(1 \rightarrow 3)$-β-D-glucan sensitive hemolymph clotting system, which seems to be important for the encapsulation and elimination of invaders, and to be linked with phagocytosis. On the other hand, the ascidians have a sophisticated lectin-complement system that occupies a pivotal role in host defense. Further understanding of innate immune systems from the comparative biochemical and genetic points of view, and careful evaluations of their homologous characters will undoubtedly provide an overview of ancestral defense mechanisms.

Added in Proof

Quite recently, Zhu *et al.* (2005) reported that a functional homolog of vertebrate complement of C3 was isolated from the horseshoe crab, *Carcinos rpius rotundicauda*. This C3 resembles human C3 and shows closest homology to C3 sequences of lower deuterostomes, Additionally, they identified the complement C2/Bf, a homolog of vertebrate C2 and factor B (Bf) that participates in C3 activation. Thus, a primitive yet complex opsonic complement defense system is revealed in the horseshoe crab, a protostome species.

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