CMLS, Cell. Mol. Life Sci. 61 (2004) 1257–1265
1420-682X/04/111257-09
DOI 10.1007/s00018-004-3396-5

CMLS Cellular and Molecular Life Sciences

Review

Structure and function of coagulogen, a clottable protein in horseshoe crabs

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Received 27 October 2003; received after revision 25 November 2003; accepted 1 December 2003

Abstract. Mammalian blood coagulation is based on the proteolytically induced polymerization of fibrinogens. Initially, fibrin monomers noncovalently interact with each other. The resulting homopolymers are further stabilized when the plasma transglutaminase (TGase) intermolecularly cross-links ε-(γ-glutamyl)lysine bonds. In crustaceans, hemolymph coagulation depends on the TGase-mediated cross-linking of specific plasma-clotting proteins, but without the proteolytic cascade. In horseshoe crabs, the proteolytic coagulation cascade triggered by lipopolysaccharides and β-1,3-glucans leads to the conversion of coagulogen into coagulin, resulting in noncovalent coagulin homopolymers through head-to-tail interaction. Horseshoe crab TGase, however, does not cross-link coagulins intermolecularly. Recently, we found that coagulins are cross-linked on hemocyte cell surface proteins called proxins. This indicates that a cross-linking reaction at the final stage of hemolymph coagulation is an important innate immune system of horseshoe crabs.

Key words. Hemolymph coagulogen; horseshoe crab; transglutaminase; lipopolysaccharides.

Introduction

Horseshoe crabs, whose ancestors were trilobites, are phylogenetically more related to arachnids than to crustaceans. They are called living fossils because the morphology of their exoskeleton is very similar to that found in Jurassic deposits. The survival of multicellular organisms depends on effective defense systems to recognize and eliminate foreign microorganisms. The major host defense system in the horseshoe crab *Tachypleus tridentatus* is carried by a hemolymph that contains one type of granular hemocyte that comprises 99% of all hemocytes [1]. The granular hemocyte is filled with two types of secretory granules, L-granules and S-granules, which selectively store defense molecules such as coagulation factors, protease inhibitors, lectins and antimicrobial peptides [2–6]. The hemocyte is highly sensitive to lipopolysaccharides (LPSs), which are cell wall components of Gram-negative bacteria. Stimulation by LPSs prompts exocytosis, which causes the excretion of defense molecules. The coagulation cascade of horseshoe crabs is composed of a clottable protein coagulogen [7–14] and four serine protease zymogens, including factor C [15–21], factor B [22, 23], factor G [24–28] and the proclotting enzyme [29, 30]. Factor C and factor G, respectively, function as biosensors for LPS and β-1,3-glucans of cell wall components of fungi; these sensors trigger the sequential activation of the coagulation factors leading to the conversion of coagulogen to coagulin. The resulting coagulins interact with each other to form homopolymers through self-polymerization. Here we focus on recent advances in the structure and function of coagulogen and discuss its role in innate immunity.

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Comparison of coagulation systems between horseshoe crabs and mammals

Compared with the mammalian coagulation system [31], the horseshoe crab system has a clotting enzyme that corresponds to thrombin, and its coagulogen corresponds to fibrinogen (fig. 1). The mammalian coagulation cascade is not a systemic reaction but proceeds locally on the phospholipid surface in cooperate with calcium ions at the site of injury. Also in horseshoe crabs, coagulation proceeds only on the surfaces of invading pathogens, such as LPSs and β-1,3-glucans. This close analogy between the coagulation system of horseshoe crabs and that of mammals may lead to a false idea of a common evolutionary origin. In fact, a fibrinogen homologue of horseshoe crabs, named tachylectin-5, has been found in plasma, and it functions as a non-self-recognizing protein rather than as a target protein of the coagulation cascade [32, 33].

On the other hand, another arthropod protease cascade has been well characterized at the molecular level as the morphogenetic cascade for determining embryonic dorsal-ventral polarity in the fly *Drosophila melanogaster*, leading to the production of a Toll ligand spaetzle, has been well characterized (fig. 1) [34]. The *Drosophila* Toll pathway also controls resistance to fungal and Gram-positive bacterial infections. Likewise in mammals, pathogen-associated molecular patterns are recognized through cell-surface receptors called Toll-like receptors [35, 36]. The structural similarity of horseshoe crab coagulogen to *Drosophila* spaetzle, as well as the sequence homology between the serine proteases participating in the two cascades, clearly suggests that these two functionally different cascades may have a common origin [14, 37–39]. The NH_2-terminal domains of factor B and the proclotting enzyme of horseshoe crabs each contain a compact 'clip' domain having three disulfide bonds. This domain has been found not only in *Drosophila* snake and easter but also in protease zymogens involved in insect prophenoloxidase activation systems [23, 30, 40–43].

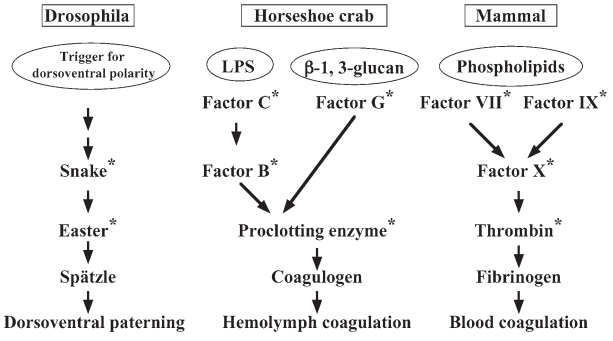

Figure 1. Comparison of proteolytic cascades between horseshoe crab hemolymph coagulation, mammalian blood coagulation and *Drosophila* dorsal-ventral patterning. The functional similarity of the coagulation cascades between mammals and horseshoe crabs is demonstrated by the self-aggregation reactions of their target proteins and the similar catalytic domains of the serine proteases. In spite of the quite different functions of the cascades of *Drosophila* and horseshoe crabs, the components of these two cascades have significant structural similarity. The target proteins coagulogen and spaetzle seem to share a common NGF-like fold in their C-terminal regions. Moreover, the serine protease zymogens easter, snake, proclotting enzyme and factor B contain a common structural motif, called a clip domain, in addition to their similar trypsin-like catalytic domains. The serine protease zymogens are indicated by stars.

In crustaceans, too, such as lobster and crayfish, hemolymph coagulation depends on the TGase-mediated cross-linking of a specific plasma-clotting protein without any proteolytic cascades. Crustacean clottable proteins have been found in several species; the freshwater crayfish *Pacifastacus leniusculus* [44], the sand crayfish *Ibacus ciliatus* [45], and the lobster *Panulirus interruptus* [46, 47]. These clottable proteins are homodimeric glycoproteins of ~380–400 kDa and have similar amino acid compositions and NH_2-terminal sequences. The crayfish clottable protein, a very high density dimeric lipoprotein consisting of 210-kDa subunits, is covalently cross-linked intermolecularly by TGase [44, 48]. The TGase-dependent coagulation is induced when TGase is released from hemocytes or tissues by a mechanism that is still unknown. Calcium ions in plasma activate the secreted TGase [49]. The polymerization of the clottable protein has been shown using electron microscopy after the purified clottable protein is incubated with hemocyte lysates containing endogenous TGase [50]. Crustacean clottable proteins do not share any sequence similarity with fibrinogen or coagulogen, whereas they are homologous to insect vitellogenins [50, 51]. Crayfish clottable protein is present in the clotting activities of both sexes. A vitellogenin specific to female crayfish has been isolated and partially characterized; it seems to differ functionally from the clottable protein [52]. Both crustacean clottable proteins and insect vitellogenins have a cysteine-containing stretch with sequence similarity to the D domain of the von Willebrand factor (vWF) [53]. vWF is a large multimeric protein involved in the mammalian blood coagulation system, and the D domain is important for multimer formation [54, 55].

In contrast to the coagulation system of horseshoe crabs, that of insects is activated as part of the wound response to avoid loss of hemolymph, even in the absence of microbial cell wall substances [56, 57]. Insect hemolymph coagulation requires interaction between plasma components (the hemolymph coagulogen) and cellular components derived from hemocytes (the hemocyte coagulogen) [58, 59]. Several plasma components involved in hemolymph coagulation have been purified; a lipid-carrying protein called lipophorin from the cockroach *Leucophaea maderae* [60]; a vitellogenin-like protein from the locust *Locusta migratoria* [46]; hemofibrin and scolexin, which are a serine protease homologue, from the tobacco hornworm *Manduca sexta* [61–63]; and a he-

mocytin with significant sequence similarity to vWF and that is from the silkworm *Bombyx mori* [64]. In several insects, lipophorin has been identified as a hemolymph coagulogen [60, 65–67]. Although the molecular mechanism of coagulation in insects remains to be clarified, these plasma proteins are possibly cross-linked with themselves or other proteins by hemocyte-derived TGase at the site of injury [68]. On the other hand, microparticles produced by the fragmentation of hemocytes are involved in the regulation of cell attachment and in the formation of hemocyte coagulogen [69]. Microparticles are enriched in hemomucin, a hemocyte surface protein. In vitro, lipophorin binds to hemomucin, and the interaction of hemomucin on microparticles with lipophorin possibly promotes hemolymph coagulation.

Self-polymerization of coagulin through head-to-tail interaction

At the final stage of the coagulation cascade of horseshoe crabs, the clotting enzyme cleaves coagulogen (175 amino acid residues) into a two-chain form of coagulin at Arg^{18}-Thr^{19} and Arg^{46}-Gly^{47}, with excision of fragment called peptide C [7–11]. Crystal structural analyses of coagulogen have revealed an elongated molecule of ~60 × 30 × 20 Å (fig. 2) [14, 37]. A striking topologic similarity has been found between the COOH-terminal half domain of coagulogen and mouse nerve growth factor (NGF), a member of the neurotrophin family. A sequence alignment based on topological equivalence shows a sequence identity of 21% in the topologically equivalent regions.

The three-dimensional structure of coagulogen suggests a possible polymerization mechanism, by which the release of the helical peptide C would expose a hydrophobic cove on the 'head'; this cove would interact with the hydrophobic edge or 'tail' of a second molecule, resulting in formation of a coagulin homopolymer (fig. 3). We have recently obtained evidence that the polymerization of coagulin proceeds through an interaction between the hydrophobic cove on the head and the hydrophobic tail [70]. The two lysine residues at positions 85 and 156, located at the head and tail regions of the elongated molecule, are chemically cross-linked intermolecularly with disuccinimidyl suberate having an arm length of 6.4 Å.

Coagulin-coagulin interaction does not require Ca^{2+} or Mg^{2+}. A spectrophotometer can be used to monitor the time course over which the clotting enzyme or trypsin converts coagulogen to coagulin, leading to homopolymers. An octapeptide containing Tyr 136, which occupies

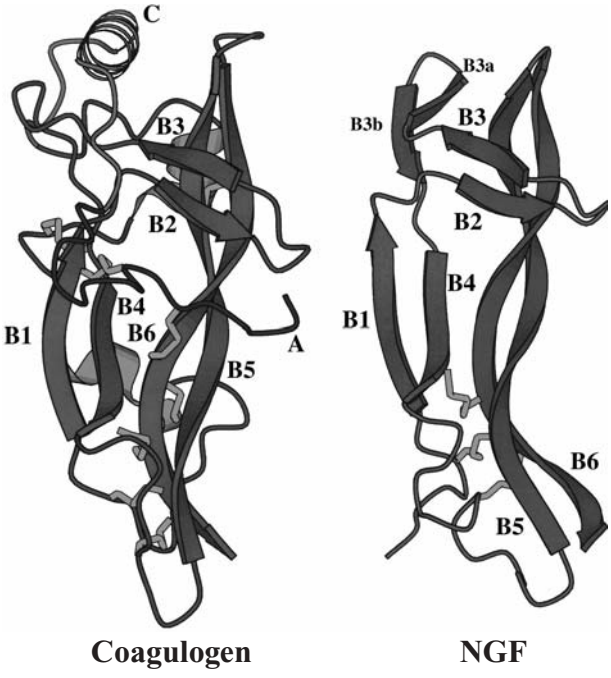

Figure 2. Comparison of three-dimensional structures of coagulogen and NGF. In coagulogen, the six β strands, B1–B6, form three antiparallel β sheets, which are topologically equivalent to β sheets in NGF. The three disulfide bridges forming the characteristic cystine knot in NGF are equivalent to those in coagulogen; thus we can assign coagulogen as a member of the cystine knot superfamily. C, peptide C; A, A chain of coagulogen.

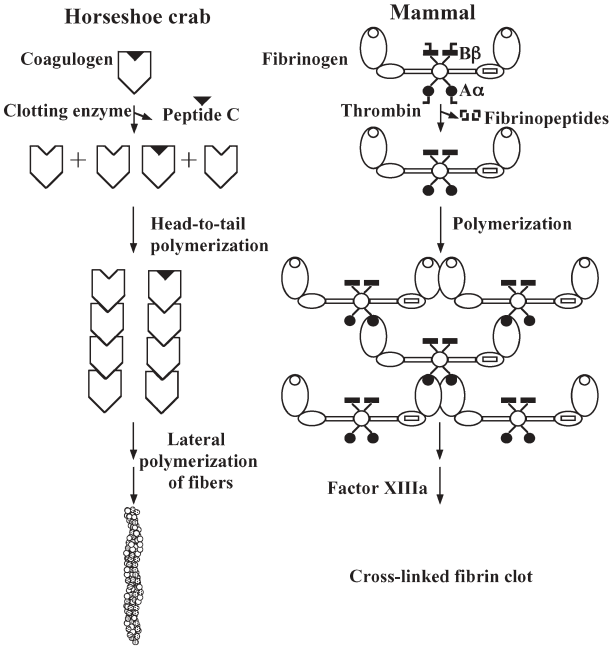

Figure 3. Comparison of a molecular model of the polymerization of coagulogen and that of fibrinogen. A putative coagulin monomer lacking peptide C may initiate polymerization through head-to-tail interaction. See the text for details. Mammalian fibrinogen consists of three homologous chains covalently assembled into $\alpha_2\beta_2\gamma_2$. At the final stage of blood coagulation, thrombin cleaves the NH_2-terminal portion of α chain, creating a new NH_2-terminus (the A site) beginning with the sequence of Gly-Pro-Arg. The A site binds to the complementary polymerization pocket in the γ chain during the alignment of the fibrin protofibrils [110–112].

the tail end of coagulin, inhibits polymerization, and the replacement of Tyr 136 of the peptide with Ala results in a loss of inhibitory activity. The polymerization of coagulin possibly proceeds through an interaction between the newly exposed hydrophobic cove on the head and the wedge-shaped hydrophobic tail.

The hydrophobic tail portion of coagulogen seems to have the same conformation as that of coagulin, and the tail region of coagulogen could interact with the hydrophobic head of coagulin to form heterodimers. Surface plasmon resonance analysis showed that coagulogen interacts with coagulin immobilized on the sensor chip through the tail of coagulogen and the head of coagulin with $K_d = 6.0 \times 10^{-6}$ M. Therefore, not only the coagulin monomer but also coagulogen could be incorporated into a coagulin fiber, then converted to coagulin by the clotting enzyme, leading to an extension of the fiber. Relative to this finding, if the serine proteases in the coagulation cascade are scavenged by the horseshoe crab serine protease inhibitors [71–73], coagulogen could regulate the extension of the fiber that will bind to the terminus.

Protein cross-linking of coagulin to cell surface proteins named proxins

No TGase activity has been found in horseshoe crab plasma, whereas horseshoe crab TGase (HcTGase) is expressed in various tissues and is mainly localized in cytosol of hemocytes [74, 75]. HcTGase is functionally and structurally similar to the mammalian type II TGase. It contains 764 amino acid residues in total with a unique NH_2-terminal extension sequence of 60 residues without a consensus NH_2-terminal signal sequence for secretion [75]. Hemocytes release HcTGase into the extracellular fluid in response to stimulation by LPSs, but the molecular mechanism of the secretion remains unknown [76]. HcTGase, however, does not catalyze coagulin-coagulin cross-linking. On the other hand, Wilson et al. reported that clots of the whole hemolymph of the American horseshoe crab *Limulus polyphemus* yield significant amounts of cross-linked products by HcTGase [77]. Therefore, coagulin is possibly cross-linked with other proteins.

Recently, we identified the proline-rich cell surface antigens on horseshoe crab hemocytes. These antigens serve as substrates for protein cross-linking with coagulin [76]. All of the monoclonal antibodies prepared against hemocytes react with the same antigens of proline-rich proteins. These antigens have been called proxins, as they are proline-rich proteins for protein cross-linking. There are two proxins, proxin-1 (271 residues) and proxin-2 (284 residues) with 66% sequence identity with each other. Proxins are constituents of major cell surface antigens of hemocytes. Immunoblotting and reverse-transcription polymerase chain reaction (PCR) have found proxins be present in hemocytes only, and not in other tissues, such as those of the heart, skeletal muscle, hepatopancreas or stomach. The intriguing feature of these sequences is the presence of four tandem repeats, each having an extremely high content of proline (accounting for 20% of the total residues). A Gln-rich domain is localized at the NH_2-terminal and the COOH-terminal regions of proxin-1, and at the NH_2-terminal regions of proxin-2 (fig. 4).

Figure 4. Domain structures of proxin-1 and proxin-2. Proxins contain four tandem-repeats of Pro-rich domains in green. The glutamine-rich regions in red are present at the NH_2-terminal regions of proxins-1 and -2 and the COOH-terminal region of proxin-1.

Proxins on hemocytes detected by fluorescence microscopy are shown in figure 5A. Although HcTGase does not cross-link coagulogen or coagulin, it promotes cross-linking of coagulin, not coagulogen, with proxins, resulting in the high molecular weight products that have been located at the top of the gel by 1% agarose gel electrophoresis in the presence of SDS [76]. Even in the absence of HcTGase, proxins noncovalently bind to coagulin but not coagulogen coated on microtiter plates, suggesting that proxins have a specific binding affinity to coagulin. At the final stage of hemolymph coagulation, HcTGase could cross-link coagulin polymers with proxins on the surface, resulting in aggregates of hemocytes entangled with coagulin polymers (fig. 5B). In the absence of HcTGase, hemocytes can easily be washed out from the coagulin polymers on slide glass. Coagulin fibers have a tendency to aggregate laterally to form a thicker fiber with a diameter of ~100 Å, probably

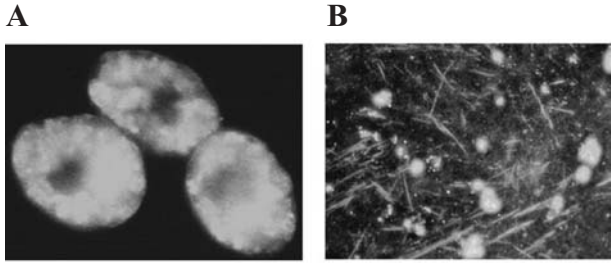

Figure 5. Cross-linking of proxins with coagulin visualized by immunofluorescence staining. (*A*) The fixed hemocytes by paraformaldehyde were incubated with monoclonal anti-proxin antibody (1 µg/ml), followed by fluorescein-conjugated secondary antibody. (*B*) The fixed hemocytes were incubated with coagulin in the presence of HcTGase and Ca^{2+}, followed by incubation with polyclonal anti-coagulogen antibody, and the coagulin fiber and hemocytes were detected by fluorescein-conjugated secondary antibody.

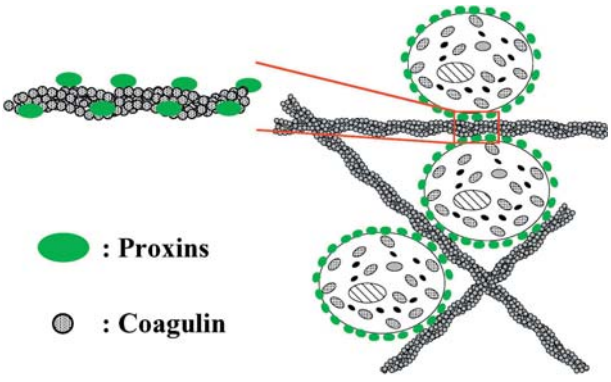

Figure 6. Hypothetical scheme of the cross-linking of coagulin with proxins on hemocytes.

through other hydrophobic patches on the coagulogen surface [14]. Another proline-rich substrate for HcTGase, tentatively named free proxin, is stored in large granules of hemocytes [6, 74]. Coagulin polymers are possibly stabilized through the HcTGase-mediated cross-linking of protein with proxins and free proxins, resulting in the formation of a reticulate structure, a casting net of an important physical barrier against invading pathogens (fig. 6).

In mammals, proline-rich proteins such as cornifins and small proline-rich proteins are involved in the formation of the cornified cell envelope, a highly insoluble structure at the cell periphery of the stratum corneum [78, 79]. The stratum corneum of the skin serves as a forefront physical barrier against invading pathogens. The envelope is composed of membrane-associated proteins, including members of the cornifin or small proline-rich protein family, in addition to several cytosolic proteins. These proteins are cross-linked into an insoluble mesh by the keratinocyte TGase, a membrane-bound enzyme. Although cornifins have no significant sequence similarity to proxins, they do consist of an NH_2-terminal glutamine-rich portion and proline-containing tandem repeats of octa- or nona-peptide [80]. Cornifins function as amine acceptors through glutamine residues of the NH_2-terminal portion. A similar glutamine cluster is also present at the NH_2-terminal regions of proxins-1 and -2 as well as in the COOH-terminal region of proxin-1 (fig. 4). Possibly, these glutamine residues of proxins function as amine acceptors. HcTGase cross-linked proxins with coagulin, but did not catalyze monodansylcadaverine incorporated into coagulin. This finding indicated that glutamine residues functioning as amine acceptors are not present on coagulin, but that several lysine residues on coagulin function as amine donors for protein cross-linking with proxins.

Conclusion

All mammalian coagulation factors circulate in plasma, and the coagulation cascade is triggered by the interaction of coagulation factor VII with a self-protein tissue factor, exposed at the site of injury. At the final stage of the coagulation reaction, noncovalently associated fibrins are further stabilized through the intermolecular cross-linking of ε-(γ-glutamyl) lysine bonds either with those fibrins themselves or with other proteins by coagulation factor XIIIa, which is essential for normal hemostasis and wound healing [31]. In contrast, all horseshoe crab coagulation factors are stored in the granules of hemocytes that are secreted in response to stimulation by LPSs. The proteolytic cascade is triggered by the interaction of coagulation factor C or factor G with non-self substances of cell wall components derived from invading pathogens. This clearly indicates that the coagulation system of horseshoe crabs is closely linked not only to hemostasis and wound healing but also to innate immunity (fig. 7). The cross-linked coagulin gel may hinder the spread of

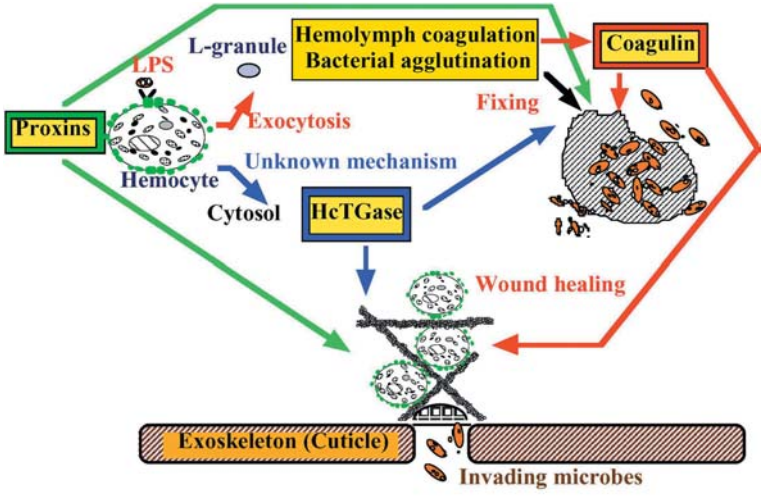

Figure 7. Innate immune system of horseshoe crabs.

the invading pathogens by immobilization, in addition to preventing the leakage of hemolymph at the site of injury. The immobilized invaders could be recognized by several lectins [32, 81–85] and subsequently killed by antimicrobial proteins [86–101].

Recently, we observed that the coagulation cascade is linked to prophenoloxidase activation in the horseshoe crab immune system [102, 103]. The horseshoe crab coagulation cascade promotes prophenoloxidase activation through the nonenzymatic interaction of factor B or proclotting enzyme with hemocyanin, which leads to the functional conversion of hemocyanin to phenoloxidase. Phenoloxidases in arthropods participate in wound healing and in repairing damaged exoskeleton, and they harden the exoskeleton during molting [104, 105]. Protease zymogens involved in prophenoloxidase activation have been identified from crustaceans and insects, which are homologous to two horseshoe crab coagulation factors: factor B and proclotting enzyme [23, 30, 40–42]. In crustaceans and insects, an ancestral protease cascade corresponding to the bifunctional cascade found in horseshoe crabs may have evolved into an exclusive system of prophenoloxidase activation.

In *Drosophila*, the Toll pathway functions as an innate immune system, but it does not function as a pattern-recognition receptor for cell wall components of invading microbes. This is because *Drosophila* Toll is thought to be activated by the ligand spaetzle, which is proteolytically produced through an upstream event in the immune response [106]. As in *Drosophila*, horseshoe crab Toll probably does not function as a pattern-recognition receptor, and the most possible candidate for a ligand is coagulin, a structural homologue of spaetzle [107]. Horseshoe crab Toll is expressed in various tissues as well as on hemocytes [unpublished data]. The molecular mechanism underlying the activation of Toll by spaetzle is not known. However, many membrane receptors are known to be activated through ligand-induced oligomerization [108, 109]. It seems possible that spaetzle induces oligomerization of *Drosophila* Toll, leading to activation of cytoplasmic signal transduction. Ideally, a coagulin oligomer may function as a ligand for horseshoe crab Toll, which induces the gene expression of proteins to restore granular components of hemocytes; this oligomer also induces the expression of wound-healing proteins to accelerate the restoration of tissues and exoskeleton at the site of injury.

Acknowledgments. This work was supported by a Grant-in-Aid for Scientific Research in Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan (to S.K.).

1 Toh Y., Mizutani A., Tokunaga F., Muta T. and Iwanaga S. (1991) Morphology of the granular hemocytes of the Japanese horseshoe crab *Tachypleus tridentatus* and immunocytochemical localization of clotting factors and antimicrobial substances. Cell Tissue Res. **266:** 137–147
2 Iwanaga S., Kawabata S. and Muta T. (1998) New types of clotting factors and defense molecules found in horseshoe crab hemolymph: their structures and functions. J. Biochem. (Tokyo) **123:** 1–15
3 Iwanaga S. (2002) The molecular basis of innate immunity in the horseshoe crab. Curr. Opin. Immunol. **14:** 87–95
4 Iwanaga S. (1993) The limulus clotting reaction. Curr. Opin. Immunol. **5:** 74–82
5 Muta T. and Iwanaga S. (1996) The role of hemolymph coagulation in innate immunity. Curr. Opin. Immunol. **8:** 41–47
6 Shigenaga T., Takayenoki Y., Kawasaki S., Seki N., Muta T., Toh Y. et al. (1993) Separation of large and small granules from horseshoe crab (*Tachypleus tridentatus*) hemocytes and characterization of their components. J. Biochem. **114:** 307–316
7 Srimal S., Miyata T., Kawabata S. and Iwanaga S. (1985) The complete amino acid sequence of coagulogen isolated from Southeast Asian horseshoe crab, *Carcinoscorpius rotundicauda*. J. Biochem. **98:** 305–318
8 Nakamura S., Iwanaga S., Harada T. and Niwa M. (1976) A clottable protein (coagulogen) from amoebocyte lysate of Japanese horseshoe crab (*Tachypleus tridentatus*). Its isolation and biochemical properties. J. Biochem. **80:** 1011–1021
9 Nakamura S., Takagi T., Iwanaga S., Niwa M. and Takahashi K. (1976) Amino acid sequence studies on the fragments produced from horseshoe crab coagulogen during gel formation: homologies with primate fibrinopeptide B. Biochem. Biophys. Res. Commun. **72:** 902–908
10 Takagi T., Hokama Y., Miyata T., Morita T. and Iwanaga S. (1984) Amino acid sequence of Japanese horseshoe crab (*Tachypleus tridentatus*) coagulogen B chain: Completion of the coagulogen sequence. J. Biochem. **95:** 1445–1457
11 Miyata T., Hiranaga M., Umezu M. and Iwanaga S. (1984) Amino acid sequence of the coagulogen from *Limulus polyphemus* hemocytes. J. Biol. Chem. **259:** 8924–8933
12 Miyata T., Usui K. and Iwanaga S. (1984) The amino acid sequence of coagulogen isolated from southeast Asian horseshoe crab, *Tachypleus gigas*. J. Biochem. **95:** 1793–1801
13 Miyata T., Matsumoto H., Hattori M., Sakaki Y. and Iwanaga S. (1986) Two types of coagulogen mRNAs found in horseshoe crab (*Tachypleus tridentatus*) hemocytes: Molecular cloning and nucleotide sequences. J. Biochem. **100:** 213–220
14 Bergner A., Oganessyan V., Muta T., Iwanaga S., Typke D., Huber R. et al. (1996) Crystal structure of limulus coagulogen: the clotting protein from horseshoe crab, a structural homologue of nerve growth factor. EMBO J. **15:** 6789–6797
15 Nakamura T., Morita T. and Iwanaga S. (1986) Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in *Limulus* hemocytes: isolation and characterization. Eur. J. Biochem. **154:** 511–521
16 Tokunaga F., Miyata T., Nakamura T., Morita T., Kuma K., Miyata T. et al. (1987) Lipopolysaccharide-sensitive serine-protease zymogen (factor C) of horseshoe crab hemocytes: Identification and alignment of proteolytic fragments produced during the activation show that it is a novel type of serine protease. Eur. J. Biochem. **167:** 405–416
17 Nakamura T., Tokunaga F., Morita T. and Iwanaga S. (1988) Interaction between lipopolysaccharide and intracellular serine protease zymogen, factor C, from horseshoe crab (*Tachypleus tridentatus*) hemocytes. J. Biochem. **103:** 370–374
18 Nakamura T., Tokunaga F., Morita T., Iwanaga S., Kusumoto S., Shiba T. et al. (1988) Intracellular serine-protease zymogen, factor C, from horseshoe crab hemocytes. Its activation by synthetic lipid A analogues and acidic phospholipids. Eur. J. Biochem. **176:** 89–94
19 Tokunaga F., Nakajima H. and Iwanaga S. (1991) Further studies on lipopolysaccharide-sensitive serine protease zymo-

gen (factor C): its isolation from *Limulus polyphemus* hemocytes and identification as an intracellular zymogen activated by alpha-chymotrypsin, not by trypsin. J. Biochem. **109:** 150–157
20 Muta T., Miyata T., Misumi Y., Tokunaga F., Nakamura T., Toh Y. et al. (1991) Limulus factor C: an endotoxin-sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like and lectin-like domains. J. Biol. Chem. **266:** 6554–6561
21 Nakamura S., Morita T., Harada-Suzuki T., Iwanaga S., Takahashi K. and Niwa M. (1982) A clotting enzyme associated with the hemolymph coagulation system of horseshoe crab (*Tachypleus tridentatus*): its purification and characterization. J. Biochem. **92:** 781–792
22 Nakamura T., Horiuchi T., Morita T. and Iwanaga S. (1986) Purification and properties of intracellular clotting factor, factor B, from horseshoe crab (*Tachypleus tridentatus*) hemocytes. J. Biochem. **99:** 847–857
23 Muta T., Oda T. and Iwanaga S. (1993) Horseshoe crab coagulation factor B: a unique serine protease zymogen activated by cleavage of an Ile-Ile bond. J. Biol. Chem. **268:** 21384–21388
24 Morita T., Tanaka S., Nakamura T. and Iwanaga S. (1981) A new (1,3)-β-D-glucan-mediated coagulation pathway found in *Limulus* amebocytes. FEBS Lett. **129:** 318–321
25 Kakinuma A., Asano T., Torii H. and Sugino Y. (1981) Gelation of *Limulus* amebocyte lysate by an antitumor (1,3)-β-D-glucan. Biochem. Biophys. Res. Commun. **101:** 434–439
26 Muta T., Seki N., Takaki Y., Hashimoto R., Oda T., Iwanaga A. et al. (1995) Purified horseshoe crab factor G: reconstitution and characterization of the (1,3)-β-D-glucan-sensitive serine protease cascade. J. Biol. Chem. **270:** 892–897
27 Seki N., Muta T., Oda T., Iwaki D., Kuma K., Miyata T. et al. (1994) Horseshoe crab (1,3)-β-D-glucan-sensitive coagulation factor G. A serine protease zymogen heterodimer with similarities to β-glucan-binding proteins. J. Biol. Chem. **269:** 1370–1374
28 Takaki Y., Seki N., Kawabata S., Iwanaga S. and Muta T. (2002) Duplicated binding sites for (1,3)-β-D-glucan in the horseshoe crab coagulation factor G. J. Biol. Chem. **277:** 14281–14287
29 Nakamura T., Morita T. and Iwanaga S. (1985) Intracellular proclotting enzyme in limulus (*Tachypleus tridentatus*) hemocytes: its purification and properties. J. Biochem. **97:** 1561–1574
30 Muta T., Hashimoto R., Miyata T. Nishimura H., Toh Y. and Iwanaga S. (1990) Proclotting enzyme from horseshoe crab hemocytes: cDNA cloning, disulfide locations and subcellular localization. J. Biol. Chem. **265:** 22426–22433
31 Furie B. and Furie B. C. (1988) The molecular basis of blood coagulation. Cell **53:** 505–518
32 Gokudan S., Muta T., Tsuda R., Koori K., Kawahara T. Seki N. et al. (1999) Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. Proc. Natl. Acad. Sci. USA **96:** 10086–10091
33 Kairies N., Beisel H.-G., Fuentes-Prior P., Tsuda R., Muta T., Iwanaga S. et al. (2001) The 2.0-Å crystal structure of tachylectin 5A provides evidence for the common origin of the innate immunity and the blood coagulation systems. Proc. Natl. Acad. Sci. USA **98:** 13519–13524
34 Belvin M. P. and Anderson K. V. (1996) A conserved signaling pathway – the *Drosphila* toll-dorsal pathway. Annu. Rev. Cell. Dev. Biol. **12:** 393–416
35 Lemaitre B., Nicolas E. Michaut L., Reichhart J. M. and Hoffmann J. A. (1996) The dorsoventral regulatory gene cassette *spaetzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. Cell **86:** 973–983
36 Rutschmann S., Kilinc A. and Ferrandon D. (2002) Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in *Drosophila*. J. Immunol. **168:** 1542–1546
37 Bergner A., Muta T., Iwanaga S., Beisel H.-G., Delotto R. and Bode W. (1997) Horseshoe crab coagulogen is an invertebrate protein with a nerve growth factor-like domain. Biol. Chem. **378:** 283–287
38 Smith C. L. and DeLotto R. (1992) A common domain within the proenzyme regions of the *Drosophila* snake and easter proteins and *Tachypleus* proclotting enzyme defines a new subfamily of serine proteases. Protein Sci. **1:** 1225–1226
39 Smith C. L. and DeLotto R. (1994) Ventralizing signal determined by protease activation in *Drosophila* embryogenesis. Nature **368:** 548–551
40 Lee S. Y., Cho M. Y., Hyun J. H., Lee K. M. Homma K. I., Natori S. et al. (1998) Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3-beta-glucan in coleopteran insect, *Holotrichia diomphalia* larvae. Eur. J. Biochem. **257:** 615–621
41 Jiang H. B., WangY. and Kanost M. R. (1998) Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta* – a bacteria-inducible protein similar to *Drosophila* easter. Proc. Natl. Acad. Sci. USA **95:** 12220–12225
42 Satoh D., Horii A., Ochiai M. and Ashida M. (1999) Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*. Purification, characterization and cDNA cloning. J. Biol. Chem. **274:** 7441–7453
43 Jiang H. B. and Kanost M. R. (2000) The clip-domain family of serine proteinases in arthropods. Insect Biochem. Mol. Biol. **30:** 95–105
44 Kopácek P., Hall M. and Söderhäll K. (1993) Characterization of a clotting protein, isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*. Eur. J. Biochem. **213:** 591–597
45 Komatsu M. and Ando S. (1998) A very-high-density lipoprotein with clotting ability from hemolymph of sand crayfish, *Ibacus ciliatus*. Biosci. Biotechnol. Biochem. **62:** 459–463
46 Doolittle R. F. and Riley M. (1990) The amino-terminal sequence of lobster fibrinogen reveals common ancestry with vitellogenins. Biochem. Biophys. Res. Commun. **167:** 16–19
47 Doolittle R. F. and Fuller G. M. (1972) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies on lobster fibrinogen and fibrin. Biochim. Biophys. Acta **263:** 805–809
48 Hall M., van Heusden M. C. and Söderhäll K. (1995) Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. Biochem. Biophys. Res. Commun. **216:** 939–946
49 Sritunyalucksana K. and Söerhäll K. (2000) The proPO and clotting system in crustaceans. Aquaculture **191:** 53–69
50 Hall M., Wang R., van Antwerpen R., Sottrup-Jensen L. and Söderhäll K. (1999) The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc. Natl. Acad. Sci. USA **96:** 1965–1970
51 Yeh M. S., Huang C. J., Leu J. H., Lee Y. C. and Tsai I. H. (1999) Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (*Penaeus monodon*). Eur. J. Biochem. **266:** 624–633
52 Sappington T. W. and Raikhel A. S. (1997) Molecular characteristics of insect vitellogenins and vitellogenin receptors. Insect Biochem. Mol. Biol. **28:** 277–300
53 Sadler J. E. (1991) von Willebrand factor. J. Biol. Chem. **266:** 22777–22780
54 Voorberg J., Fontijn R., van Mourik J. A. and Pannekoek H. (1990) Domains involved in multimer assembly of von Willebrand factor (vWF): multimerization is independent of dimerization. EMBO J. **9:** 797–803
55 Mayadas T. N. and Wagner D. D. (1992) Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. Proc. Natl. Acad. Sci. USA **89:** 3531–3535

56 Bohn H. (1986) Hemolymph clotting in insects. In: Immunity in Invertebrates, Brehélin M. (ed.), pp. 189–207, Springer, Heidelberg

57 Theopold U., Li D., Fabbri M., Scherfer C. and Schmidt O. (2002) The coagulation of insect hemolymph. Cell. Mol. Life Sci. **59:** 363–372

58 Bohn H., Barwig B. and Bohn B. (1981) Immunochemical analysis of hemolymph clotting in the insect *Leucophaea maderae* (Blattaria). J. Comp. Physiol. **143:** 169–184

59 Barwig B. and Bohn H. (1980) Evidence for the presence of two clotting proteins in insects. Naturwissenschaften **67:** 47

60 Barwig B. (1985) Isolation and characterization of plasma coagulogen (PC) of the cockroach *Leucophaea maderae* (Blattaria). J. Comp. Physiol. **155:** 135–143

61 Geng C. and Dunn P. E. (1988) Hemostasis in larvae of *Manduca sexta*: formation of a fibrous coagulum by hemolymph proteins. Biochem. Biophys. Res. Commun. **155:** 1060–1065

62 Minnick M. F., Rupp R. A. and Spence K. D. (1986) A bacterial-induced lectin which triggers hemocyte coagulation in *Manduca sexta*. Biochem. Biophys. Res. Commun. **137:** 729–735

63 Finnerty C. M., Karplus P. A. and Granados R. R. (1999) The insect immune protein scolexin is a novel serine proteinase homolog. Protein Sci. **8:** 242–248

64 Kotani E., Yamakawa M., Iwamoto S., Tashiro M., Mori H., Sumida M. et al. (1995) Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. Biochim. Biophys. Acta **1260:** 245–258

65 Brehélin M. (1979) Hemolymph coagulation in *Locusta migratoria*: evidence for a functional equivalent of fibrinogen. Comp. Biochem. Physiol. **62:** 329–334

66 Gellissen G. (1983) Lipophorin as the plasma coagulogen in *Locusta migratoria*. Naturwissenschaften **70:** 45–46

67 Duvic B. and Brehélin M. (1998) Two major proteins from locust plasma are involved in coagulation and are specifically precipitated by laminarin, a β-1,3-glucan. Insect Biochem. Mol. Biol. **28:** 959–967

68 Li D., Scherfer C., Korayem A. M., Zhao Z., Schmidt O. and Theopold U. (2002) Insect hemolymph clotting: evidence for interaction between the coagulation system and the prophenoloxidase activating cascade. Insect Biochem. Mol. Biol. **32:** 919–928

69 Theopold U. and Schmidt O. (1997) *Helix pomatia* lectin and annexin V, two molecular probes for insect microparticles: possible involvement in hemolymph coagulation. J. Insect Physiol. **43:** 667–674

70 Kawasaki H., Nose T., Muta T., Iwanaga S., Shimohigashi Y. and Kawabata S. (2000) Head-to-tail polymerization of coagulin, a clottable protein of the horseshoe crab. J. Biol. Chem. **275:** 35297–35301

71 Miura Y., Kawabata S. and Iwanaga S. (1994) A limulus intracellular coagulation inhibitor with characteristics of the serpin superfamily. Purification, characterization and cDNA cloning. J. Biol. Chem. **269:** 542–547

72 Miura Y., Kawabata S., Wakamiya Y., Nakamura T. and Iwanaga S. (1995) A limulus intracellular coagulation inhibitor type 2. Purification, characterization, cDNA cloning and tissue localization. J. Biol. Chem. **270:** 558–565

73 Agarwala K. L., Kawabata S., Miura Y., Kuroki Y. and Iwanaga S. (1996) Limulus intracellular coagulation inhibitor type 3. Purification, characterization, cDNA cloning and tissue localization. J. Biol. Chem. **271:** 23768–23774

74 Tokunaga F., Yamada M., Miyata T., Ding Y. L., Hiranaga-Kawabata M., Muta T. et al. (1993) Limulus hemocyte transglutaminase. Its purification and characterization and identification of the intracellular substrates. J. Biol. Chem. **268:** 252–261

75 Tokunaga F., Muta T., Iwanaga S., Ichinose A. Davie E. W., Kuma K. et al. (1993) Limulus hemocyte transglutaminase. cDNA cloning, amino acid sequence and tissue localization. J. Biol. Chem. **268:** 262–268

76 Osaki T., Okino N., Tokunaga F., Iwanaga S. and Kawabata S. (2002) Proline-rich cell surface antigens of horseshoe crab hemocytes are substrates for protein cross-linking with a clotting protein coagulin. J. Biol. Chem. **277:** 40084–40090

77 Wilson J., Rickles F. R., Armstrong P. B. and Lorand L. (1992) N-epsilon(gamma-glutamyl)lysine crosslinks in the blood clot of the horseshoe crab, *Limulus polyphemus*. Biochem. Biophys. Res. Commun. **188:** 655–661

78 Steinert P. M. and Marekov L. N. (1995) The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. J. Biol. Chem. **270:** 17702–17711

79 Robinson N. A., Lapic S., Welter J. .F. and Eckert R. L. (1997) S100A11, S100A10, annexin I, desmosomal proteins, small proline-rich proteins, plasminogen activator inhibitor-2 and involucrin are components of the cornified envelope of cultured human epidermal keratinocytes. J. Biol. Chem. **272:** 12035–12046

80 Austin S. J., Fujimoto W., Marvin K. W., Vollberg T. M., Lorand L. and Jetten A. M. (1996) Cloning and regulation of cornifin β, a new member of the cornifin/spr family. Suppression by retinoic acid receptor-selective retinoids. J. Biol. Chem. **271:** 3737–3742

81 Saito T., Kawabata S., Hirata M. and Iwanaga S. (1995) A novel type of limulus lectin-L6. Purification, primary structure and antibacterial activity. J. Biol. Chem. **270:** 14493–14499

82 Okino N., Kawabata S., Saito T., Hirata M., Takagi T. and Iwanaga S. (1995) Purification, characterization and cDNA cloning of a 27-kDa lectin (L10) from horseshoe crab hemocytes. J. Biol. Chem. **270:** 31008–31015

83 Inamori K., Saito T., Iwaki D., Nagira T., Iwanaga S., Arisaka F. et al. (1999) A newly identified horseshoe crab lectin with specificity for blood group A antigen recognizes specific O-antigens of bacterial lipopolysaccharides. J. Biol. Chem. **274:** 3272–3278

84 Saito T., Hatada M., Iwanaga S. and Kawabata S. (1997) A newly identified horseshoe crab lectin with binding specificity to O-antigen of bacterial lipopolysaccharides. J. Biol. Chem. **272:** 30703–30708

85 Iwaki D., Osaki T., Mizunoe Y., Wai S. N., Iwanaga S. and Kawabata S. (1999) Functional and structural diversities of C-reactive proteins present in horseshoe crab hemolymph plasma. Eur. J. Biochem. **264:** 314–332

86 Tanaka S., Nakamura T., Morita T. and Iwanaga S. (1982) *Limulus* anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of *Limulus* coagulation system. Biochem. Biophys. Res. Commun. **105:** 717–723

87 Ohashi K., Niwa M., Nakamura T., Morita T. and Iwanaga S. (1984) Anti-LPS factor in the horseshoe crab, *Tachypleus tridentatus*. Its hemolytic activity on the red blood cell sensitized with lipopolysaccharide. FEBS Lett. **176:** 207–210

88 Morita T., Ohtsubo S., Nakamura T., Tanaka S., Iwanaga S., Ohashi K. et al. (1985) Isolation and biological activities of *Limulus* anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). J. Biochem. **97:** 1611–1620

89 Nakamura T., Furunaka H., Miyata T., Tokunaga F., Muta T., Iwanaga S. et al. (1988) Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Isolation and chemical structure. J. Biol. Chem. **263:** 16709–16713

90 Miyata T., Tokunaga F., Yoneya T., Yoshikawa K., Iwanaga S., Niwa M. et al. (1989) Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II, and polyphemusins I and II: chemical structures and biological activity. J. Biochem. **106:** 663–668

91 Muta T., Fujimoto T., Nakajima H. and Iwanaga S. (1990) Tachyplesins isolated from hemocytes of Southeast Asian horseshoe crabs (*Carcinoscorpius rotundicauda* and *Tachypleus gigas*): identification of a new tachyplesin, tachyplesin III, and a processing intermediate of its precursor. J. Biochem. **108:** 261–266

92 Shigenaga T., Muta T., Toh Y., Tokunaga F. and Iwanaga S. (1990) Antimicrobial tachyplesin peptide precursor. cDNA cloning and cellular localization in the horseshoe crab (*Tachypleus tridentatus*). J. Biol. Chem. **265:** 21350–21354

93 Muta T., Nakamura T., Furunaka H., Tokunaga F., Miyata T., Niwa M. et al. (1990) Primary structures and functions of anti-lipopolysaccharide factor and tachyplesin peptide found in horseshoe crab hemocytes. Adv. Exp. Med. Biol. **256:** 273–285

94 Katsu T., Nakao S. and Iwanaga S. (1993) Mode of action of an antimicrobial peptide, tachyplesin I, on biomembranes. Biol. Pharm. Bull. **16:** 178–181

95 Hoess A., Watson S., Siber G. R. and Liddington R. (1993) Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor, at 1.5 Å resolution. EMBO J. **12:** 3351–3356

96 Kawano K., Yoneya T., Miyata T., Yoshikawa K., Tokunaga F., Terada Y. et al. (1990) Antimicrobial peptide, tachyplesin I, isolated from hemocytes of the horseshoe crab (*Tachypleus tridentatus*): NMR determination of the β-sheet structure. J. Biol. Chem. **265:** 15365–15367

97 Park N. G., Lee S., Oishi O., Aoyagi H., Iwanaga S., Yamashita S. et al. (1992) Conformation of tachyplesin I from *Tachypleus tridentatus* when interacting with lipid matrices. Biochemistry **31:** 12241–12247

98 Saito T., Kawabata S., Shigenaga T., Takayenoki Y., Cho J., Nakajima H. et al. (1995) A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence and antibacterial activity. J. Biochem. **117:** 1131–1137

99 Kawabata S., Nagayama R., Hirata M., Shigenaga T., Agarwala K. L., Saito T. et al. (1996) Tachycitin, a small granular component in horseshoe crab hemocytes, is an antimicrobial protein with chitin-binding activity. J. Biochem. **120:** 1253–1260

100 Osaki T., Omotezako M., Nagayama R., Hirata M., Iwanaga S., Kasahara J. et. al. (1999) Horseshoe crab hemocyte-derived antimicrobial polypeptides, tachystatins, with sequence similarity to spider neurotoxins. J. Biol. Chem. **274:** 26172–26178

101 Kawabata S., Tokunaga F., Kugi Y., Motoyama S., Miura Y., Hirata M. et al. (1996) Limulus factor D, a 43-kDa protein isolated from horseshoe crab hemocytes, is a serine protease homologue with antimicrobial activity. FEBS Lett. **398:** 146–150

102 Nagai T. and Kawabata S. (2000) A link between blood coagulation and prophenol oxidase activation in arthropod host defense. J. Biol. Chem. **275:** 29264–29267

103 Nagai T., Osaki T. and Kawabata S. (2001) Functional conversion of hemocyanin to phenoloxidase by horseshoe crab antimicrobial peptides. J. Biol. Chem. **276:** 27166–27170

104 Ratcliffe N. A., Rowley A. F., Fitzgerald S. W., Rhodes C. P. and (1985) Invertebrate immunity: basic concepts and recent advances. Int. Rev. Cytol. **97:** 183–350

105 Sugumaran M. (1996) Roles of the insect cuticle in host defense reactions. In: New Directions in Invertebrate Immunology, pp. 355–374, Söderhäll K., Iwanaga S. and Vasta G. R. (eds), SOS Publications, Fair Haven

106 Levashina E. A., Langley E., Green C., Gubb D., Ashburner M., Hoffmann J. A. et al. (1999) Constitutive activation of Toll-mediated antifungal defense in serpin-deficient *Drosophila*. Science **285:** 1917–1919

107 Inamori K., Koori K. Mishima C., Muta T. and Kawabata S. (2000) A horseshoe crab receptor structurally related to *Drosophila* Toll. J. Endotoxin Res. **6:** 397–399

108 Lemmon M. A. and Schlessinger J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. Trends Biochem. Sci. **19:** 459–463

109 Mizuguchi K., Parker J. S., Blundell T. L. and Gay N. J. (1998) Getting knotted: a model for the structure and activation of Spaetzle. Trends Biochem. Sci. **23:** 239–242

110 Pratt K. P., Cote H. C., Chung D. W., Stenkamp R. E. and Davie E. W. (1997) The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal γ chain fragment complexed with the peptide Gly-Pro-Arg-Pro. Proc. Natl. Acad. Sci. USA **94:** 7176–7181

111 Spraggon G., Everse S. J. and Doolittle R. F. (1997) Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. Nature **389:** 455–462

112 Brown J. H., Volkmann N., Jun G., Henschen-Edman A. H. and Cohen C. (2000) The crystal structure of modified bovine fibrinogen. Proc. Natl. Acad. Sci. USA **97:** 85–90