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MOLECULAR MECHANISM OF HEMOLYMPH CLOTTING SYSTEM IN *LIMULUS*

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ABSTRACT *Limulus* (horseshoe crab) hemolymph is known to be very sensitive to bacterial endotoxin (LPS), which causes a rapid coagulation response. Hemolymph contains a single type of hemocyte that undergoes aggregation, adhesion, and degranulation in response to LPS. The granule contents are released into the hemolymph, where they form an insoluble gel. We have characterized four components involved in this coagulation response that comprise a cascade of three serine protease zymogens (factor C, factor B, and proclotting enzyme) and one clottable protein (coagulogen). Of these components, factor C sensitive to LPS is a protein composed of five complement-related domains ("Sushi" or SCR), an EGF-like domain, and a C-type lectinlike domain as well as a putative amino-terminal LPS-binding domain. This domain structure is very similar to that of selectin family of cell adhesion molecules, suggesting that it might also function as a cell adhesion molecule after the release into the hemolymph. Factor B and the proclotting enzyme share a common Cys-rich motif ("cliplike" domain) in the amino-terminal portions. This domain is also found in a putative serine protease zymogen ("easter") in *Drosophila*, which is essential for normal embryonic development. All four of the components of the cascade and an antibacterial protein (anti-LPS factor) are localized to a specific type of the hemocyte granule. Another antibacterial peptide (tachyplesins I and II) is localized in a distinct granule population. The contents of both granule populations are released into the hemolymph in response to LPS, where they cooperate in immobilization and killing of Gram-negative bacteria.

INTRODUCTION

Invertebrates have characteristic host defense systems that differ from mammalian immune systems (1–6). In *Limulus* (horseshoe crab), an *Arthropoda* species, this defense system is carried by the hemolymph, which contains a type of cell called an amebocyte. The amebocytes

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are extremely sensitive to endotoxin (LPS), which is a major component of the cell surface of Gram-negative bacteria (7–11). When hemolymph comes into contact with Gram-negative bacteria or LPS, the amebocytes begin to degranulate, and hemolymph coagulation is initiated by the granule components (12–17). This response is thought to be very important for limulus not only in preventing the leakage of hemolymph, but also in engulfing invading microorganisms (18–20).

During the past decade, we have studied the molecular mechanism of hemolymph coagulation in limulus and have established a protease cascade, as shown in Figure 1. This cascade is based on three kinds of serine protease zymogens, factor C, factor B, proclotting enzyme, and one clottable protein, coagulogen. LPS activates the zymogen factor C to the active factor $\bar{C}$. Factor $\bar{C}$ then activates factor B to factor $\bar{B}$, which in turn converts the proclotting enzyme to the clotting enzyme. Each activation proceeds by limited proteolysis. The resulting clotting enzyme cleaves two bonds in coagulogen, which is a fibrinogen-like molecule in arthropods, to yield an insoluble coagulin gel. The coagulation cascade is also activated by (1,3)-β-D-glucan, a serine protease that is tentatively named factor G is activated, leading to activation of the proclotting enzyme.

In this review, we will focus on the molecular structures and functions of limulus clotting factors and antimicrobial substances so far studied in our laboratory.

Limulus Hemocytes

The limulus hemolymph contains two types of cells, granular hemocytes and cyanocytes (12,14–17,20). Based on cell morphology, there appears to be only one type of hemocyte in the systemic circulation of the adult intermolt animal, the so-called granulocyte or amebocyte (4). This cell is an oval, plate-shaped structure, 15–20 μm in its longest dimension. Figure 2 shows an electron micrograph of the hemocyte separated from Japanese horseshoe crab, *Tachypleus (T) tridentatus* (20). The cell contains numerous dense granules classed into two major types: large (L) and small (S) granules. The L-granules are larger (up to 1.5 μm in diameter) and less electron dense than the S-granules (<0.6 μm in diameter). The L-granules contain at least 20 protein components including, 4 clotting factors and 1 antimicrobial factor (anti-LPS factor), whereas the S-granules exclusively contain the other antimicrobial substance, named tachyplesin peptide, in addition to 6 major protein components. After

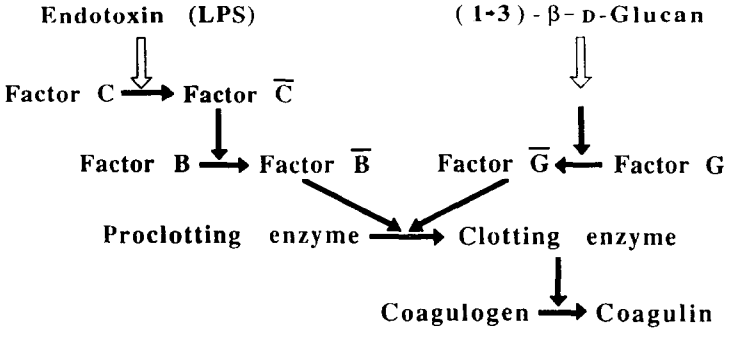


FIG. 1.

Hemolymph coagulation cascade system in *Limulus* (*Tachypleus tridentatus*)

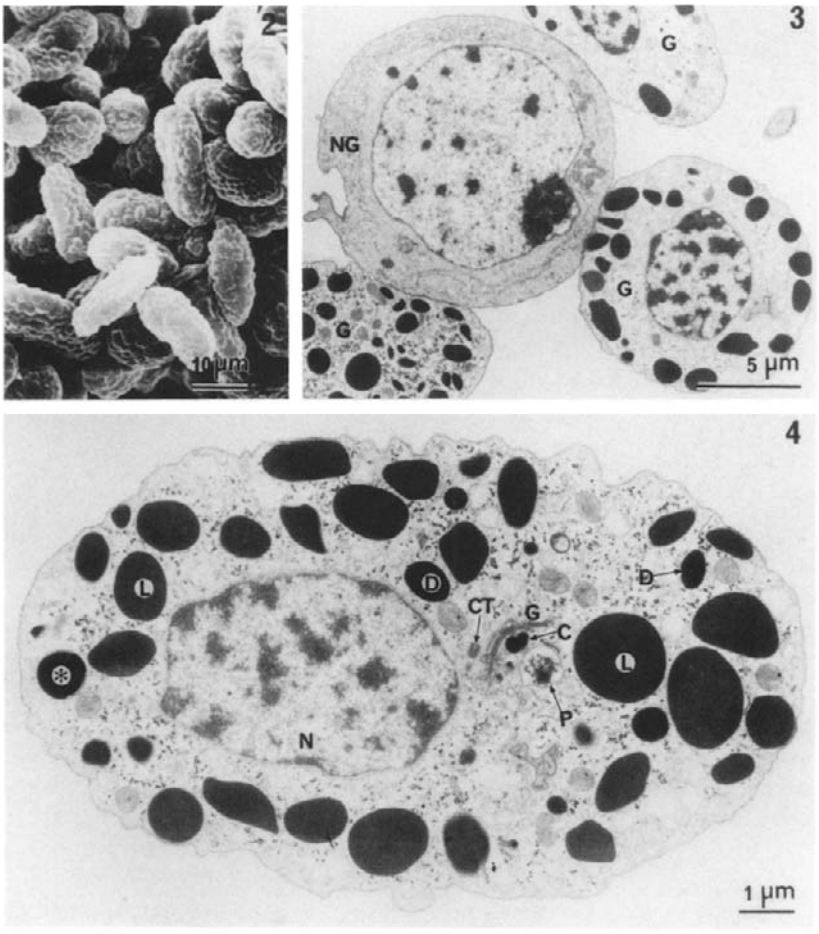


FIG. 2.

A. A scanning electron micrograph of hemocytes; **B.** Granular hemocytes (G) and non-granular hemocytes (NG); **C.** A cross-section of a granular hemocyte showing the distribution of various organelles. **C.** golgi vacuole with condensed dense materials; **CT.** centriole; **D.** dense (small) granules; **G.** golgi complex; **L.** large granules; **P.** golgi vacuole with dispersed dense materials (P-granule). Published by permission of Springer-Verlag, Heidelberg. Original in Ref. 20.

treatment with endotoxin or LPS, the L-granules are released more rapidly than the S-granules, although almost all granules are finally exocytosed (20). This exocytosis is associated with clot formation, the process being complete within 90 seconds. The clot is more soft than mammalian fibrin clot and contains coagulin gel generated from its precursor, named coagulogen.

Coagulogen

The clottable protein, coagulogen, has a functional similarity with vertebrate fibrinogen (21–23) and is known to play a central role in the limulus clotting system (Fig. 1) (24–29). In the

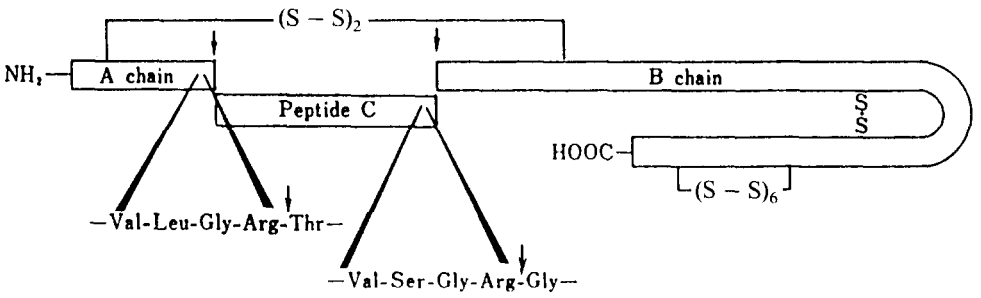


FIG. 3.

The gross structure of *Limulus* coagulogen. The arrows indicate sites cleaved by *Limulus* clotting enzyme in the conversion of coagulogen to coagulin gel.

intact animal, soluble precursor of this protein is absent from the plasma, but is instead sequestered in the cytoplasmic L-granules of the hemocytes (14,20). This granule exocytosis releases coagulogen into the external milieu (12,15). The extracellular clot is composed of fibers of the protein, named coagulin (11).

Figure 3 shows a gross structure of coagulogen and its structural change by limulus clotting enzyme (30–33). Coagulogen is an invertebrate fibrinogen-like substance, and it is converted from a soluble form to an insoluble gel, coagulin. The conversion to a gel is mediated by a clotting enzyme in the hemocytes (25,26). Coagulogen consists of a single basic polypeptide chain and has a calculated molecular weight of 19,700 (±50). This protein contains three segments, A chain, peptide C, and B chain (34). The clotting enzyme cleaves the Arg–Gly and Arg–Thr linkages, both located at the NH_2-terminal region (24,25). The Arg–Gly linkage cleaved by the clotting enzyme is the same type as that cleaved by α-thrombin in the transformation of mammalian fibrinogen to fibrin. Moreover, the COOH-terminal octapeptide sequences of A chain and peptide C exhibit great homology to one another, and their sequences are very similar to that of primate fibrinopeptide B (25,32). The amino acid sequences of four coagulogens isolated from the hemocytes of American (*Limulus (L) polyphemus*), Japanese (*T. tridentatus*), and two Southeast Asian (*T. gigas* and *Carcinoscorpius (C) rotundicauda*) horseshoe crabs are shown in Fig. 4 (34–40). Upon gelation of all the coagulogens by a limulus clotting enzyme, a large peptide, named peptide C, is released in common from the inner portion of the parent molecules. The resulting gel consists of two chains of A (18 residues) and B (129 residues), bridged by two disulfide linkages. The overall sequences of the four coagulogens have considerable homology, particularly in the A and B chain regions, consisting of 147 residues (40). Surprisingly, 73%–92% of the sequences in these regions contain the same amino acid residues in identical positions, and homologies are found along the entire molecules. In addition, 16 half-cystine residues of the four are in the same positions in the sequences, suggesting that they are paired in the same fashion and, thus, that the molecules have very similar conformations. In contrast, the sequence homology of the peptide C region is less than that of the A and B chain regions. The homologies amount to approximately 43%–57% of the structure (41,42). It is of interest that coagulogen appears to contain an α-helical (14.9%) region exclusively around the peptide C segment released by the clotting enzyme (39), suggesting that there is a marked conformational change in the transformation of coagulo-

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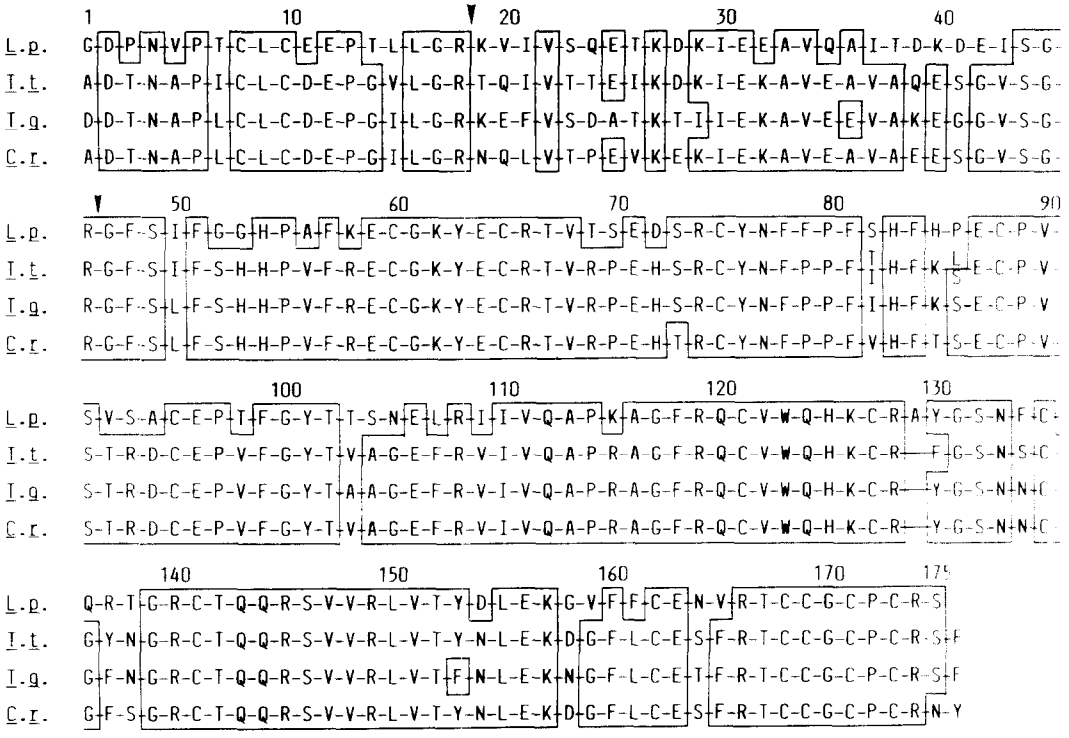


FIG. 4.

Amino acid sequence identities among Asian (*T.t.*, *T.g.*, *C.r.*) and American (*L.p.*) horseshoe crabs. Chemically identical residues in the sequences are framed. The bonds cleaved by horseshoe crab clotting enzyme are indicated by arrows. *L.p.*, *Limulus polyphemus*; *T.t.*, *Tachypleus tridentatus*; *T.g.*, *Tachypleus gigas*; *C.r.*, *Carcinoscorpius rotundicauda*. Published by permission of the Japanese Biochemical Society, Tokyo. Original in Ref. 39.

gen to coagulin gel. On the other hand, the β-sheet (24.6%), reverse-turn (29.7%), and coiled (30.9%) structure are commonly distributed in the B chain segment, suggesting that it is a β-type protein (39).

We also identified a cloned DNA fragment for coagulogen from a cDNA library of the limulus hemocyte mRNA (37). Figure 5 shows the nucleotide sequences of two precoagulogen cDNA clones and their predicted amino acid sequences. The cDNA of 946 bp encoded for precoagulogen II is found to code for a 20-bp noncoding region at the 5′ end, a leader sequence of 20 amino acids, followed by the entire coding region of coagulogen, and a 341-bp noncoding region at the 3′ end. The predicted amino acid sequence of this clone is in good agreement with the sequence determined by the protein-based study, except for the residue at positions 82 and 86, the sequence of which is identical to that found in coagulogen isolated from *T. gigas*. On the other hand, the sequence of cDNA encoded for precoagulogen I agreed completely with that previously established (38), indicating that the hemocyte coagulogens are encoded by two very similar but distinct mRNAs.

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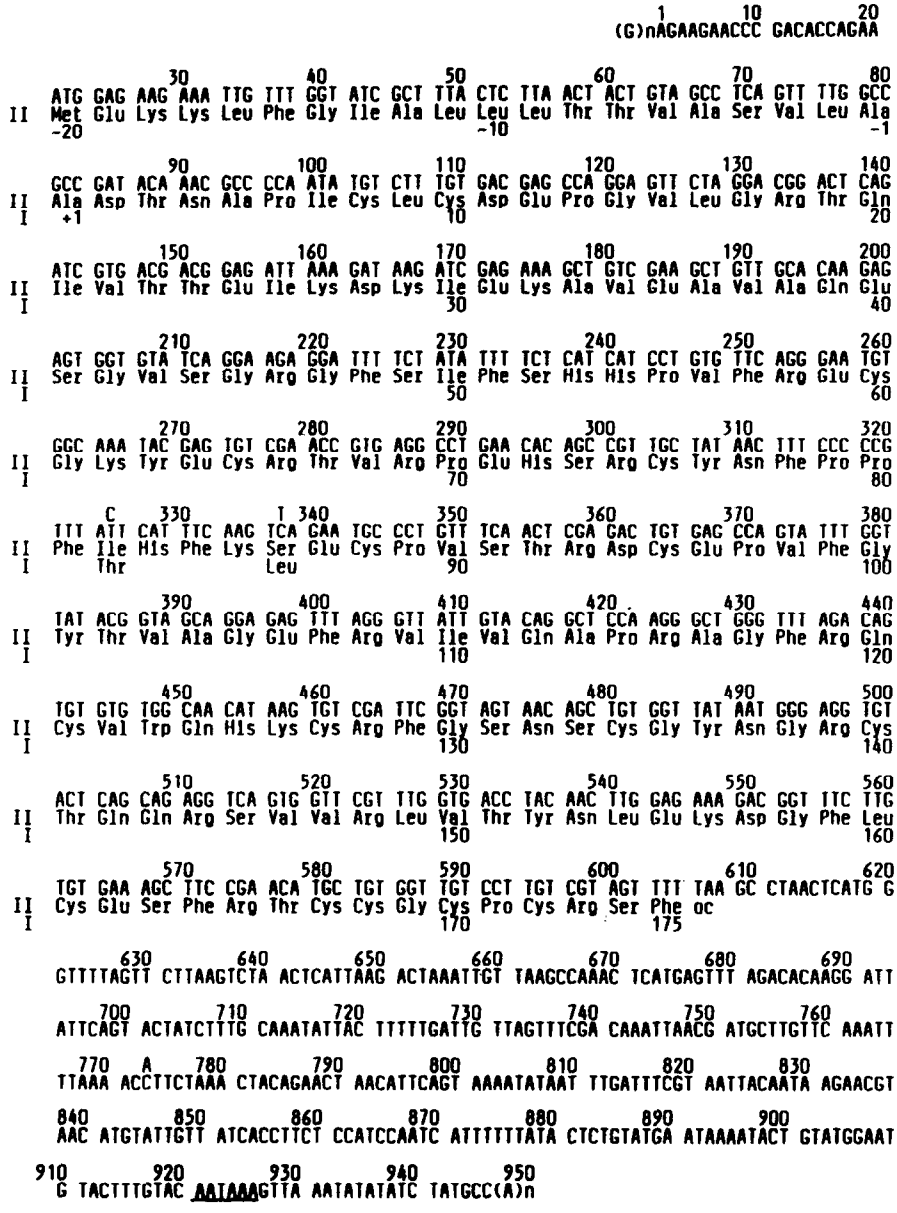


FIG. 5.

Nucleotide sequences of two precoagulogen cDNA clones, p4i and p3C21, and their predicted amino acid sequences. The numbers above and below the sequences show the nucleotide positions and the amino acid positions respectively. The positive numbers of the amino acids start from the NH_2-terminus of coagulogen. The putative poly (A) additional signal, AATAAA, is underlined. The clone p4i was 811 bp in length starting from nucleotide position 137 encoded for the amino acid residues 20-175 in coagulogen type I. The clone p3C21 was 946 bp in length starting from nucleotide position 1 encoded for coagulogen type II, (G)n and (A)n are the oligo(G) and oligo(A) residues; oc is the ochre translation stop codon TAA. Published by permission of the Japanese Biochemical Society, Tokyo. Original in Ref. 37.

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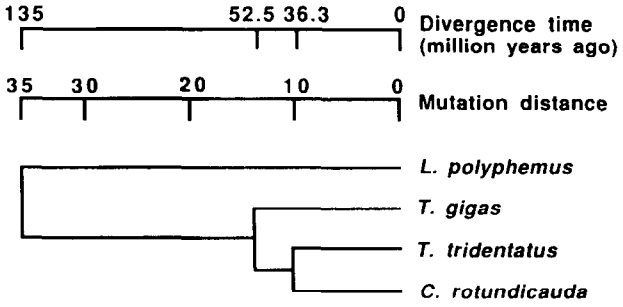


FIG. 6.

Phylogenetic relationship of living horseshoe crab species based on mutation distances calculated from the amino acid sequences of coagulogens (6). Published by permission of the Japanese Biochemical Society, Tokyo. Original in Ref. 39.

As well known, protein sequences have been used to estimate the evolutionary rate and phylogenetic relationships among different species at the molecular level (43,44). Based on the data on amino acid differences and minimum base substitutions among the sequences of coagulogens from the four species, the mutation distance between Limulinae and Tachypleinae is approximately 67.3; that between *T. gigas* and the other Asian species is 25 and that between *T. tridentatus* and *C. rotundicauda* is 20. A cladogram (based on the mutation distances among coagulogen sequences) indicating the phylogeny for the living horseshoe crab species is illustrated in Figure 6. These data agree with previous reports (32) that *T. tridentatus* and *C. rotundicauda* are phylogenetically more closely related than the other species of horseshoe crab, and the fact that *Limulus* is evolutionarily distant from the other three species is supported by the results of immunochemical studies on coagulogen (33) and sialic acid binding lectin (32). According to Shuster (43), divergence from the common ancestor of Limulinae and Tachypleinae occurred in the late Jurassic or early Cretaceous period, corresponding to 110–160 million years ago. The calculated divergence time between *T. gigas* and the other Asian species is 52.5 million years and that between *T. tridentatus* and *C. rotundicauda* is 36.3 million years. These values differ from those suggested by Shuster (44) and also from those estimated previously based on the amino acid sequences of peptide C (residues 19–46) of coagulogen (32). The approximate mutation rate per amino acid site in coagulogen per year (R) can be calculated by the method of Kimura using the following equation; $R = A/13.5 \times 10^7 \times B \times 2$. A is the average number of amino acid replacements separating Limulinae and Tachypleinae, and B is the number of amino acid residues in coagulogen. The value of 13.5×10^7 is the average time in years from the late Jurassic and early Cretaceous periods to the present. We can calculate A and B as 54.7 and 175, respectively, and, thus, R for coagulogen is 1.16×10^{-9}. This value is approximately a half of that obtained for peptide C, 2.03×10^{-9} (39,42).

Limulus Clotting Factors

Proclotting Enzyme. Limulus proclotting enzyme is activated by both factor B and factor G (Fig. 1) and it is a single chain glycoprotein with an apparent molecular mass of 54 kDa. Upon activation by factor B (Fig. 1), it is converted to a two chain active form, clotting enzyme

(31,49,50), composed of a light (L, 25 kDa) and a heavy (H, 31 kDa) chains (48,52). The clotting enzyme cleaves two bonds in coagulogen to yield a coagulin gel. Thus, proclotting enzyme/clotting enzyme seems to be a prothrombin/α-thrombin counterpart to invertebrates, because both catalyze a final step for gelation. Interestingly, however, the clotting enzyme has a substrate specificity similar to that of mammalian coagulation factor Xa. It catalyzes the conversion of bovine prothrombin to α-thrombin at a reasonable rate (48). As the activity of clotting enzyme is inhibited by several serine protease inhibitors (45–48), this protein is thought to be a serine protease zymogen (51), and it was proved, afterwards, by its primary structure (53).

Figure 7 shows cDNA and the entire amino acid sequences of proclotting enzyme (53). The sequence from the first ATG codon shows characteristics of a signal sequence. The 29 amino acid residues (amino acid -29 to -1) preceding the amino-terminal glutamine residue of mature protein seems to constitute a prepro-sequence. The hydropathy profile calculated by the method of Kyte and Doolittle indicates that the presequence region is highly hydrophobic, suggesting that this region acts as a signal peptide. The sequence linked between the mature protein and the signal peptide is assumed to consist of 12 (or 5) residues and appears to represent a propeptide region of the immature protein. In fact, the carboxyl-terminal sequence of this region shows a sequence, -Arg-X-Arg-Arg (amino acid -4 to -1) similar to those found in serine protease zymogens associated in mammalian coagulation and complement systems (Fig. 8). The presence of this sequence strongly suggests that an enzyme, which proteolytically processes the dibasic cleavage site, might also exist in the invertebrate hemocyte. The propeptide region might function so as to prevent an unexpected activation of the proclotting enzyme, the result being clot formation within the cell.

The mature protein is composed of 346 amino acid residues with a molecular mass of 38,194 (Fig. 7). The amino-terminal residue of the intact proclotting enzyme is masked by a pyroglutamyl residue. All three potential glycosylation sites, one in the L chain and two in the H chain, for *N*-linked carbohydrate chains (Asn-X-Ser/Thr) are actually glycosylated. Furthermore, at least six *O*-linked carbohydrate chains are present in the L chain. The cleavage site associated with the zymogen activation is Arg_{98}–Ile_{99}, the sequence of which connects the L and H chains. The sequence preceding this site has an unique structure, -Thr-Thr-Thr-Thr-Arg. This sequence agrees well with the substrate specificity of the limulus factor $\bar{B}$ (54). The H chain of the proclotting enzyme is composed of a typical structure of serine protease. The locations of four disulfide linkages in the H chain are the same as those in prothrombin and factor IX. As previously mentioned, clotting enzyme shows a substrate specificity similar to that of factor Xa. In fact, the entire sequence of the H chain corresponding to the serine protease region shows a close similarity with that of human factor Xa (34.1%). It contains also the His_{143}–Asp_{199}–Ser_{297} triad known to be the catalytic triad of serine proteases. The substrate binding site corresponding to Ser_{189} in chymotrypsin numbering is aspartic acid, thereby indicating that this clotting enzyme has a typical trypsinlike specificity.

The amino-terminal portion of the L chain consists of a novel disulfide-knotted structure. This structure forms a "cliplike" shape consisting of a discrete domain. Following this domain, the serine and threonine-rich sequence are highly glycosylated with *O*-linked carbohydrate chains. After we published the entire sequence of proclotting enzyme, Dr. A. Day (Dept. of Biochemistry, Univ. of Oxford) informed us that the proclotting enzyme has a sequence similarity in its amino-terminal light chain portion with *Drosophila* protein, named serine protease easter precursor. Figure 9 shows a similarity of parts of these proteins, indicating that all cysteine residues are aligned. Although the significance is still unknown, these results may

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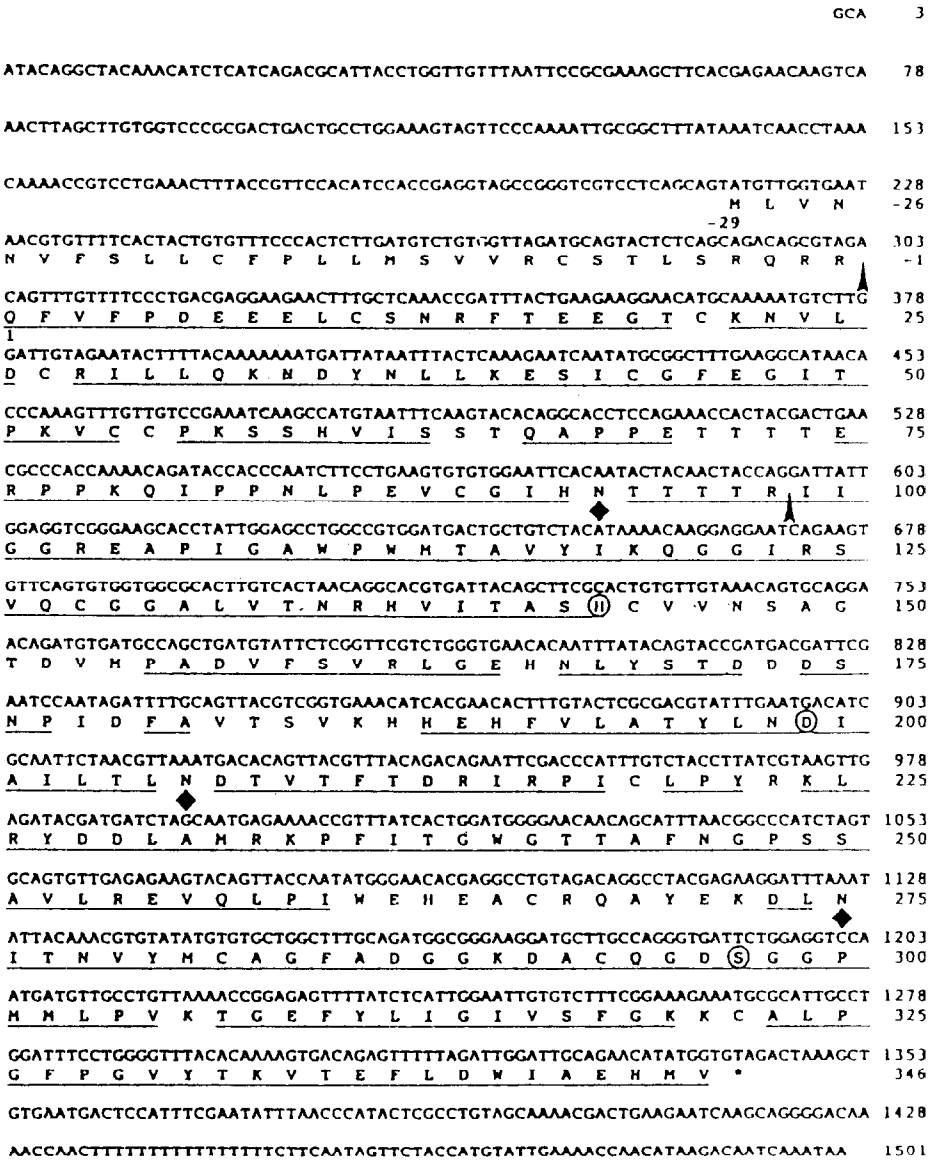


FIG. 7.

Nucleotide and deduced amino acid sequences of proclotting enzyme. Nucleotide (*upper*) and deduced amino acid sequences (*lower*) are shown. The amino acid sequences are numbered from the amino-terminus of the mature protein. The *underlined* amino acid residues were confirmed by protein sequencing. The cleavage sites accompanied by maturation and activation are shown by *arrowheads*. The potential attachment sites for *N*-linked carbohydrate chains are indicated by *closed diamonds*. The active triads of the serine protease domain are *circled*. Published by permission of the American Society for Biochemistry and Molecular Biology, Inc. (ASBMB). Original in Ref. 53.

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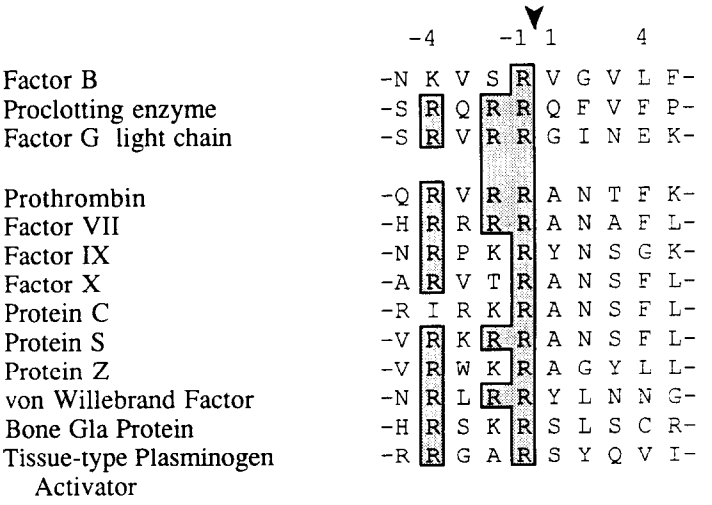


FIG. 8.

Sequence comparisons of processing sites of limulus immature clotting factors with those of immature mammalian vitamin K dependent clotting factors and others. An arrow indicates the sites cleaved by a microsomal processing enzyme (105).

suggest that the "cliplike" domain each found in limulus proclotting enzyme and factor B is one of the common structural elements in the serine protease zymogen of invertebrate animals.

An immunohistochemical study utilizing anticlotting enzyme antibody demonstrates that the proclotting enzyme is present in the L-granules in hemocytes (20).

Clotting Factor B. Factor B is activated by factor C and then, in turn, activates proclotting enzyme (Fig. 1) (55,56). Factor B is a single-chain glycoprotein with a molecular mass of 64,000 (nonreduced SDS-PAGE). The purified factor B, however, gives three bands with apparent molecular masses of 64,000, 40,000 (heavy chain), and 25,000 (light chain) after reduction (54). Therefore, the purified preparation contains two molecular species; single-chain factor B and two-chain factor B bridged by a disulfide linkage. The two-chain species appears

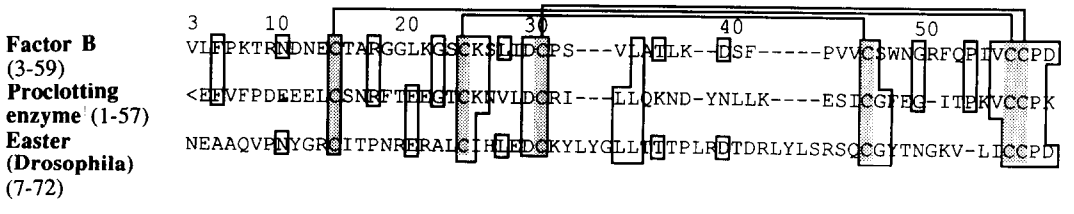


FIG. 9.

Sequence similarity of "Clip-like" domain of limulus proclotting enzyme and factor B with that of serine protease zymogen easter from *Drosophila* (106).

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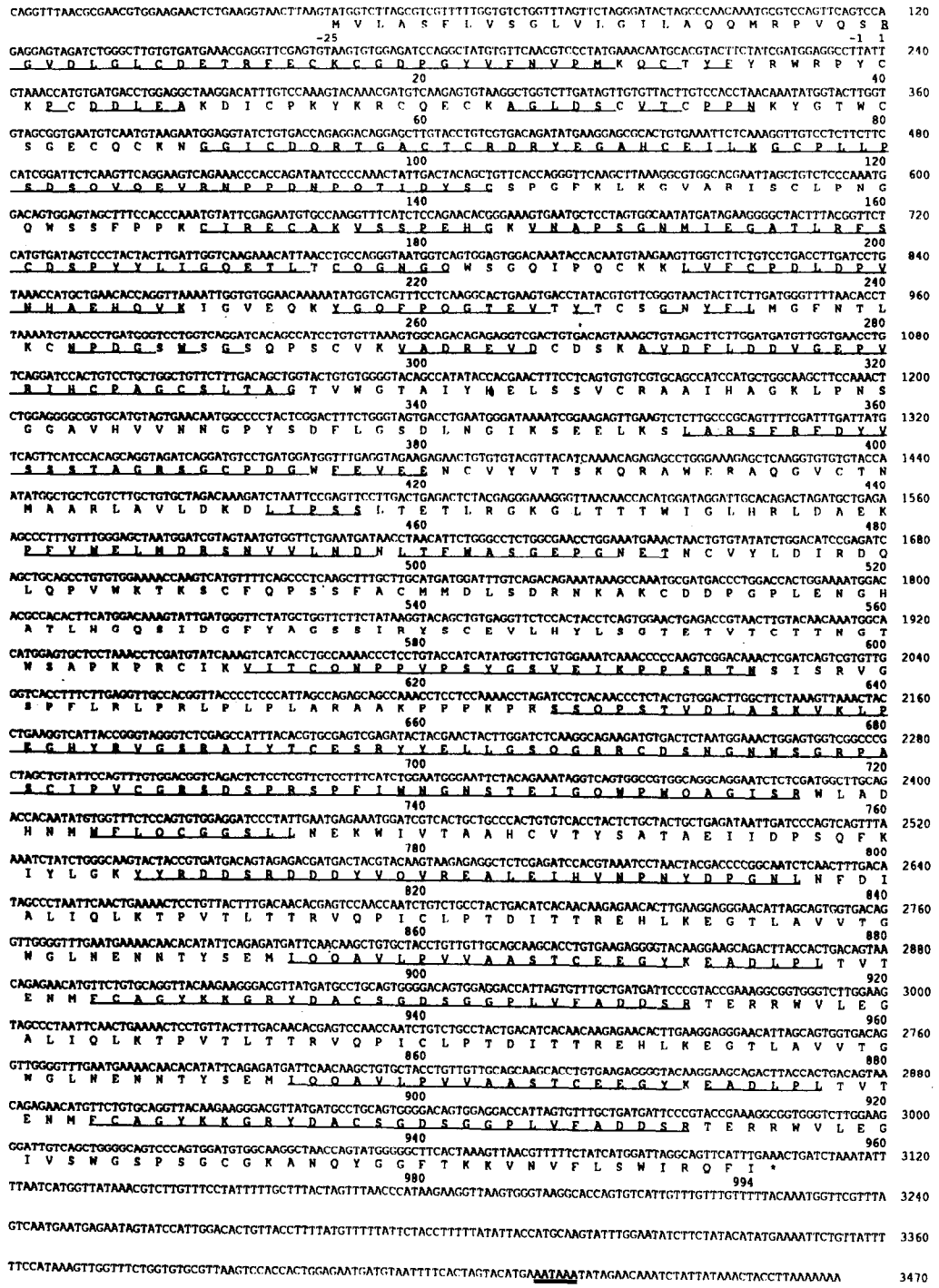
most likely to be a factor B zymogen cleaved in a disulfide loop that links the heavy and the light chains. Upon activation of the zymogen factor B by active factor $\bar{C}$, it is converted into factor $\bar{B}$ with the heavy ($Mr = 32,000$) and light chains ($Mr = 25,000$), releasing an activation peptide. When the purified factor $\bar{B}$ is treated with [^{3}H]DFP, one major peak with radioactivity before reduction is found at the position corresponding to the major protein band of factor $\bar{B}$. On reduction, the major radioactive peak migrates to the position corresponding to the mobility of the heavy chain ($Mr = 32,000$) derived from factor $\bar{B}$, indicating that the DFP-reactive site is located in the heavy chain portion of factor $\bar{B}$.

Recently, we succeeded to determine the cDNA sequence for zymogen factor B (57). It consists of 400 amino acid residues with 23 residues of signal sequence. The mature protein is composed of 377 amino acids with a calculated molecular mass of 40,570. Three potential glycosylation sites for *N*-linked carbohydrate chain are found in the mature polypeptide sequence. The entire amino acid sequence has a similarity with that of limulus proclotting enzyme. Particularly, the sequence identity of the carboxyl-terminal serine protease domains between factor B and proclotting enzyme is 43.9%. Moreover, the amino-terminal regions up to 60th residue of both proteins share a similar structure with six cysteines (Fig. 9), suggesting that these proteins are arisen from a gene duplication.

Factor $\bar{B}$ most efficiently hydrolyzes Boc-Met-Thr-Arg-4-methylcoumaryl-7-amide-(MCA) and shows a relatively weak activity towards Boc-Leu-Gly-Arg-MCA, which is the best substrate for limulus clotting enzyme (54). The good substrate (Boc-Val-Pro-Arg-MCA) for factor $\bar{C}$ is also weakly hydrolyzed by factor $\bar{B}$. Factor $\bar{B}$ shows an apparent affinity for hydroxyl amino acid (Thr or Ser) at the P_2 site (nomenclature of Schechter and Berger). As regards to natural substrates, it does not activate any mammalian plasma coagulation factors, such as factor IX, factor X, prothrombin, plasminogen, protein C, and prekallikrein. It also does not convert fibrinogen to fibrin gel. Moreover, factor $\bar{B}$ does not catalyze the activation of the zymogen factor C or the conversion of coagulogen to coagulin. The amidase activity of factor $\bar{B}$ is strongly inhibited by α_2-plasmin inhibitor, but is not inhibited by antithrombin III, which is a potent inhibitor of factor $\bar{C}$ and limulus clotting enzyme. Among four synthetic inhibitors, DFP has the strongest inhibitory effect. Benzamidine, leupeptin, and PCMB partially inhibit the factor $\bar{B}$ activity (54).

Clotting Factor C. As shown in Fig. 1, factor C is an initial activator of the clotting cascade triggered by LPS. Factor C has a molecular weight (M_r) of 123,000 glycoprotein consisting of a heavy chain of $M_r = 80,000$ and a light chain of $M_r = 43,000$ (58–60). Factor C is converted to an activated form in the presence of LPS with a $M_r = 123,000$, designated factor $\bar{C}$ (62–64). Upon activation, a single cleavage of Phe–Ile bond in the light chain occurs, resulting in the accumulation of two new fragments of $M_r = 34,000$ (B chain) and 8,500 (A chain) (62). A di*iso*propylfluorophosphate-sensitive active site is localized in the B chain of factor $\bar{C}$. We determined the entire amino acid sequence of factor C using recombinant DNA technique (65). The zymogen consists of 994 residues with a calculated molecular mass of 109,648 kDa (Fig. 10). In the H and A chains, there are several interesting amino acid sequences. First, factor C has five repeating units ("Sushi domain or short consensus repeat) of about 60 amino acid residues each, which are found in many proteins participating in the mammalian complement system. Factor C is the first example of a protein that has the complement-like structure in invertebrates. Second, an epidermal growth factor (EGF)-like domain is present in the H chain. This EGF-like domain is most homologous with that of laminin B_2 chain. Proteins containing this type of domain involve several blood coagulation factors, complement factors,

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some receptors including low density lipoprotein (LDL) receptor. Third, a lectinlike domain is unexpectedly found in between the third and fourth "Sushi" domains. This region is composed of about 120 amino acids and shows homology with so-called C-type lectins (65). This type of domain is also found in proteins whose functions are not considered only to bind the carbohydrate moiety. Any sugar binding abilities in factor C have not been demonstrated, thus far.

Two other characteristic sequences are also found in the H chain (Fig. 12). One is a Cys-rich region, which is located in the amino-terminal portion of the H chain. Eleven residues of cysteines out of 56 residues are clustered in this region (Fig. 10). The other is in the carboxyl-terminal portion of the H chain, in which 13 out of 48 residues are proline. This region has homology with the connecting region of mammalian coagulation factor XII (65). Its significance, however, remains unknown.

The B chain portion of factor C is composed of a typical serine protease, with an active site triad. The B chain shows the strongest homology with human α-thrombin (36.7%). This is consistent with the result that factor C shows a similar specificity toward synthetic fluorogenic peptide substrates comparable with α-thrombin (58). The substrate binding site of aspartic acid is conserved, which indicates that factor C is a trypsin type enzyme, although cleavage site associated with the zymogen activation is the Phe-Ile (60). The proline residue at the P_2 site might be important for the cleavage. In fact, the peptide bond cleaved during conversion to the two-chain form, Arg-Ser, is also preceded by a proline residue. The mosaic structure of factor C is summarized in Fig. 12. Immunohistochemical observation with electron microscopy utilizing anti-factor C antibody demonstrates that factor C is localized in the L-granules in the cell (20).

To our knowledge, proteases with Sushi domains are only mammalian complement C1r, C1s, C2, factor B, and limulus factor C, as shown in Fig. 11. Moreover, proteases that have both Sushi and EGF domains include only C1r, C1s, and factor C, all of which are associated with the initiation of cascade reaction. "Selectins" including three membrane proteins, endothelial leukocyte adhesion molecule, lymph node homing receptor, and granule membrane protein 140, have been reported to contain a lectinlike, an EGF-like domain and several "Sushi" domains (Fig. 12). The similarity in domain composition between factor C and those proteins is of interest, because these three proteins all seem to be associated with biologic defense systems. Another example that contain all these domains is a proteoglycan core protein

FIG. 10.

The composite nucleotide sequence (*upper*) and the deduced amino acid sequence (*lower*) of factor C. A polyadenylation signal (AATAAA) is *double-underlined.* The amino acid sequence is *numbered* beginning at the amino terminus of the mature factor C molecule. The amino acid residues that have been confirmed by sequencing of purified peptides are *underlined* and include data from Tokunaga *et al.* (60). Amino-terminal amino acid sequencing of each chain identified the three cleavage sites marked by *arrowheads.* The potential carbohydrate attachment sites for (Asn-Xaa-Ser/Thr) are indicated with *closed diamonds* and the putative attachment site confirmed not to carry carbohydrate is shown as an *open diamond.* The amino acid residues suspected to form the catalytic triad of the serine protease domain are *circled.* Published by permission of the American Society for Biochemistry and Molecular Biology, Inc. (ASBMB). Original in Ref. 65.

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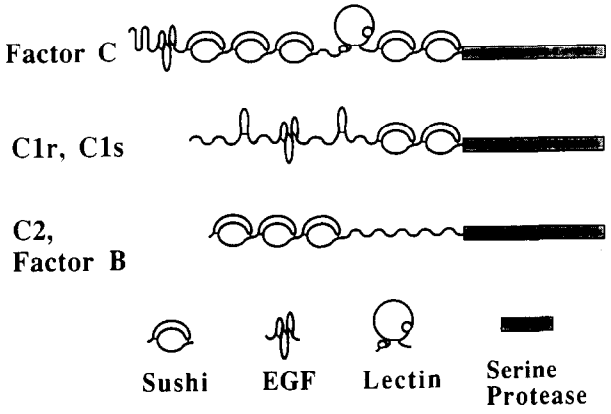


FIG. 11.

Comparison of the structures of serine proteases containing Sushi domains. The domain structures of serine proteases with Sushi domains including limulus factor C, C1r, C1s, C2, and complement factor B are illustrated. Published by permission of the American Society for Biochemistry and Molecular Biology, Inc. (ASBMB). Original in Ref. 65.

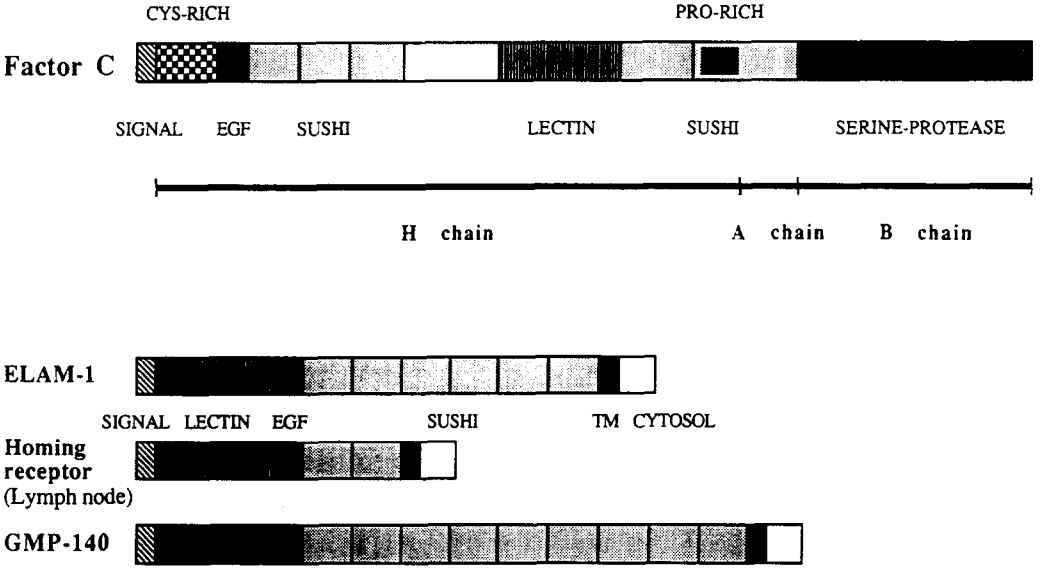


FIG. 12.

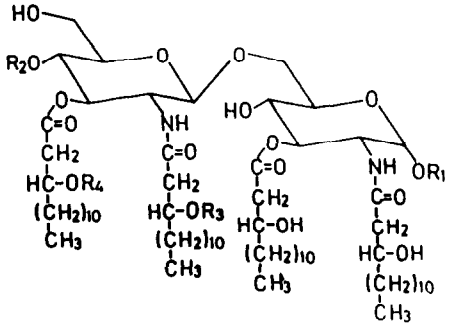
The domain structure of limulus zymogen factor C sensitive to LPS. Factor C has a mosaic structure of several domains including a signal peptide (-25 to -1), one Cys-rich region (1-76), one EGF-like domain (77-111), five Sushi domains (117-170, 174-229, 235-296, 551-609, and 618-665, located within the fifth Sushi domain) and one serine protease domain (738-994). The H, A, and B chain correspond to each domain as illustrated. These domain structures are compared with those of the so-called "selectin" family. Published by permission of the American Society for Biochemistry and Molecular Biology, Inc. (ASBMB). Original in Ref. 65.

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that is present extracellular matrix (65). On the other hand, the fact that factor C is structurally related to mammalian complement factors indicates evolutionary relationship between two systems. Factor C is a newly discovered type of serine protease zymogen, a "coagulation-complement factor," which may play an important role both in hemostasis and defense mechanisms. In considering the structural similarity of initiators of the two systems, the limulus coagulation and mammalian complement systems may have evolved from a common origin. Concerning the evolution of host defense systems in the animal kingdom, this is consistent with a possible common evolutionary origin of the coagulation and complement cascades.

The zymogen factor C is rapidly activated not only by bacterial endotoxins (58,61) but also by acylated (β1-6)-D-glucosamine disaccharide bisphosphate (synthetic *Escherichia coli*-type lipid A), and the corresponding 4′-monophosphate analogues, as shown in Tables I and II (63,64). However, the corresponding nonphosphorylated lipid A does not activate factor C, indicating that a phosphate ester group linked with the (β1-6)-D-glucosamine disaccharide back bone is required for the zymogen activation. In addition, the zymogen factor C is significantly activated by acidic phospholipids, such as phosphatidylinositol, phosphatidylglycerol, and cardiolipin, but not at all by neutral phospholipids (Table III). The rate of this activation, however, is affected markedly by ionic strength in the reaction mixture, although such an effect is not observed in the lipid-A-mediated activation of factor C. A variety of negatively charged surfaces, such as sulfatide, dextran sulfate, and ellagic acid, which are known

TABLE I
Structure of chemically synthesized lipid A analogues. Published by permission of Elsevier Science Publishers BV, Amsterdam, Holland. Original in Ref. 63.



	Substituent			
Number	R_1	R_2	R_3	R_4
403	$-H$	$-H$	$-H$	$-H$
404	$-H$	$-PO(OH)_2$		
405	$-PO(OH)_2$	$-H$		
406	$-PO(OH)_2$	$-PO(OH)_2$		
503	$-H$	$-H$	$-CO(CH_2)_{10}CH_3$	$-CO(CH_2)_{12}CH_3$
504	$-H$	$-PO(OH)_2$		
505	$-PO(OH)_2$	$-H$		
506	$-PO(OH)_2$	$-PO(OH)_2$		

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TABLE II

Activation of factor C by lipid A analogues. Synthetic lipid A analogues were dissolved in 0.025% triethylamine (1 mg/ml), sonicated at room temperature for 10 min. and diluted with 0.05 M Tris/HCl (pH 8.0) containing 0.5 mg/ml human serum albumin. As the compounds 403 and 503 were not soluble in the above buffer, their sonicated emulsions were used alone for assay. The activation of factor C (7.7 nM) at various concentrations of lipid A analogues were examined, and the minimal concentration of each lipid A analogue for 50% activation of factor C (ED_{50}) was estimated. Published by permission of Elsevier Science Publishers BV, Amsterdam, Holland. Original in Ref. 63.

Compound	Activation	Concentration of lipid A to activate 50% of factor C (ED_{50})
		nM
403	−	
404	+	16
405	+	143
406	+	6
503	−	
504	+	66
505	+	220
506	+	55

as typical initiators for activation of the mammalian intrinsic clotting system, do not show any effect on the zymogen factor C activation. These results suggest that lipid A is the most effective trigger to initiate the activation of the horseshoe crab hemolymph clotting system. In addition to nonenzymatic activators described previously, the zymogen factor C is rapidly ac-

TABLE III

Activation of factor C by phospholipids. Phospholipids were dissolved in water (1 mg/ml), sonicated at room temperature for 10 min and diluted with 0.05 M Tris/HCl (pH 8.0) containing 0.5 mg/ml human serum albumin. Then the activation of factor C (7.7 nM) at various concentrations of phospholipids was examined and the minimal concentration of each phospholipid for 50% activation of factor C was estimated, as shown in Table I. Published by permission of Elsevier Science Publishers BV, Amsterdam, Holland. Original in Ref. 63.

Phospholipids	Activation	Concentration of phospholipid to activate 50% of factor C (ED_{50})
		nM
Phosphatidic acid	+	>10000
Phosphatidylglycerol	+	22
Phosphatidylserine	+	45
Phosphatidylinositol	+	8
Phosphatidylethanolamine	−	
Phosphatidylcholine	−	
Diphosphatidylglycerol (cardiolipin)	+	24

tivated by α-chymotrypsin and rat mast cell chymase (63,66), but not by trypsin, which activates many serine protease zymogens associated with mammalian coagulation, fibrinolysis, and complement systems. Thus, limulus factor C seems to be a novel type of serine protease zymogen susceptible to α-chymotrypsin. The amidase activity of factor $\bar{C}$ toward Boc-Val-Pro-Arg-MCA is strongly inhibited by antithrombin III, but not by α_2-antiplasmin. Soybean trypsin inhibitor and hirudin has no inhibitory effect on factor $\bar{C}$ activity. DFP and D-Phe-Pro-Arg-chloromethyl ketone have a potent inhibitory effect. Benzamidine and leupeptin also have inhibitory activity at high concentrations (5 mM and 0.23 mM, respectively), while *p*-chloromercuribenzoate has no apparent inhibitory effect (58).

Figure 13 summarizes the gross structures of limulus clotting factors so far described. Like mammalian clotting factors, factor C, factor B and proclotting enzyme are typical ser-

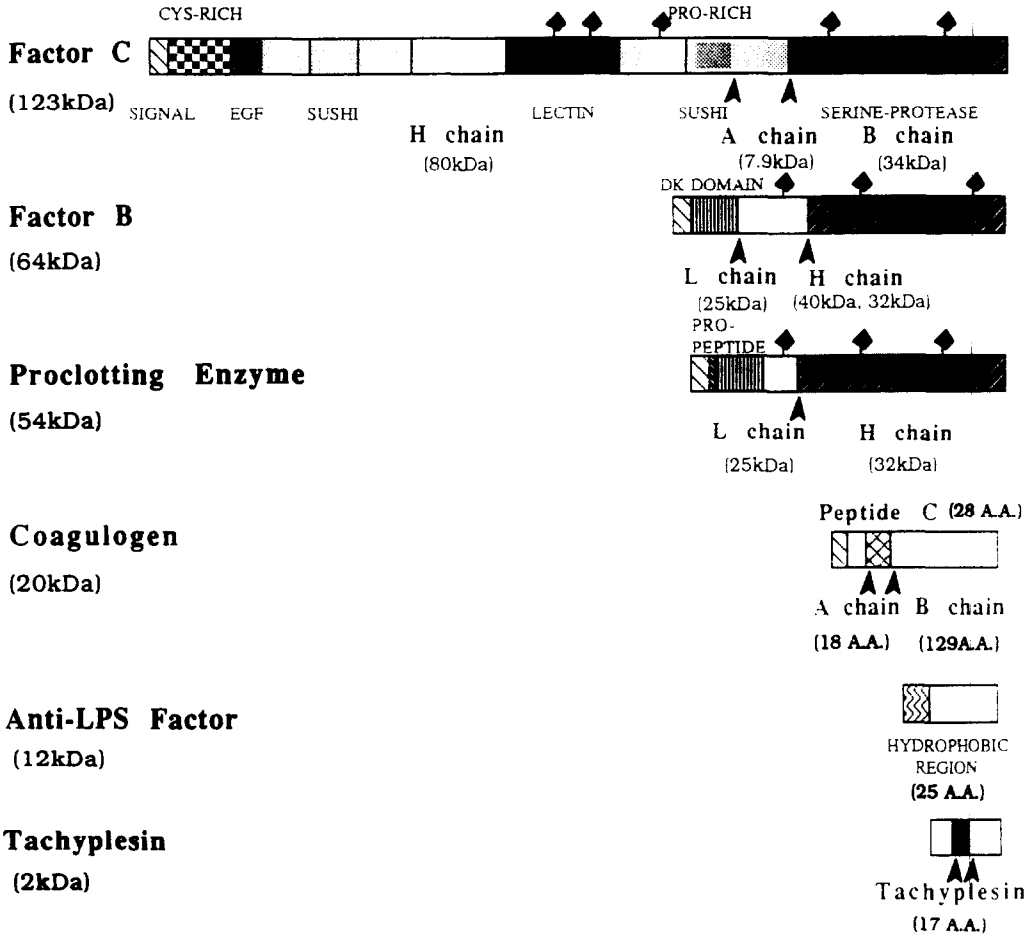


FIG. 13.

The domain structures of limulus clotting factors and antimicrobial substances. The arrowheads indicate the cleavage sites for zymogen activation. The potential carbohydrate attachment sites are indicated with closed diamonds.

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ine protease zymogens related to trypsin family. They have structural domains in their amino-terminal portions, which are different from those of mammalian clotting factors. There is no Gla domain, no kringle domain, and no finger domain. Moreover, mammalian clotting factors do not have "Sushi" domain, whereas factor C contains several "Sushi" domains like complement factors, and the proclotting enzyme and factor B contain a "cliplike" domain in the amino-terminal portions. On the other hand, coagulogen molecule is much smaller than vertebrate fibrinogen. Thus, all the limulus intracellular clotting factors are thought to be a separate entity from the vertebrate clotting factors, although there is an intracellular clotting cascade similar to that of mammals.

Antimicrobial Substances

Hemolymph circulating in horseshoe crab contains several antimicrobial substances (71–73). Two potent antimicrobial substances, anti-LPS factor and tachyplesin, have so far been found in *Tachypleus* and *Limulus* hemocytes. They have ability to bind with LPS molecule, and anti-LPS factor is localized in the L-granules together with clotting factors and tachyplesin in the S-granules (Fig. 2), both of which are secreted by the stimulation of LPS (20).

Anti-LPS factor (ALF). Primary structure of ALF isolated from Japanese horseshoe crab was first determined in 1986 (6,70,77), and then, that of American crab was established in the next year (76). Both ALF are a single-chain polypeptide composed of 101 or 102 amino acid residues with the molecular weight of 12,000. No carbohydrate is attached. The primary structures are shown in Fig. 14. Whereas the Japanese ALF has a masked amino-terminus with pyroglutamic acid, it is aspartyl residue in the American ALF. The Japanese ALF has heterogeneities of valine and isoleucine at position 36 and glutamine and glutamic acid at its carboxyl-terminus. On the other hand, American ALF contains asparagine and lysine at position 13 as microheterogeneity. Both molecules contain two cysteine residues at the same position (positions 31 and 52) linked with an intramolecular disulfide bridge (70). The sequence identity between two molecules is 83%. This value is rather higher than that obtained from comparison of coagulogen molecules from two species (69%), indicating that the entire molecule

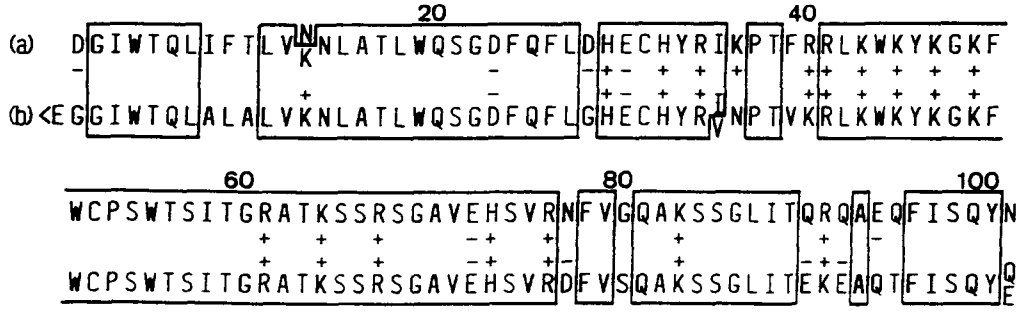


FIG. 14.

Alignment of the sequences of anti-LPS factors (ALF) from *L. polyphemus* (a) and *T. tridentatus* (b). Identical residues are boxed and charged residues are indicated by + or − between the two sequences. Published by permission of the Japanese Biochemical Society, Tokyo. Original in Ref. 76.

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is closely associated with its biologic function. In charge distribution along the polypeptide chains, the region in the disulfide loop is highly basic. Especially, the region from arginine-41 to lysine-49 has basic amino acids at every second residues, and the region from arginine-61 to arginine-76 is also rich in basic residues, in which they have positive charges almost at every third residues. Hydropathic profile indicates that amino-terminal region of ALF is highly hydrophobic, which render this molecule amphipathic (70).

ALF inhibits the LPS-mediated activation of factor C (69). Because the activation of factor C induced by synthetic lipid A is also inhibited by ALF, it appears to interact with lipid A portion of LPS. ALF also shows the hemolytic activity against red blood cells (RBC) sensitized with LPS (78). Not only human RBC but also horse and chicken RBC are susceptible to the hemolysis. The minimal concentration of ALF required for hemolysis is dependent on the concentration of LPS used for the sensitization. Under optimal conditions, the minimal hemolytic concentration of ALF is 0.48 μg/ml. The hemolysis mediated with ALF completes within 1 minute at 37°C, and even at 0°C this reaction completes within 5 minutes. The LPS sensitization is essential for these phenomenon, because ALF does not show any activities against intact cells. This activity of ALF can be neutralized by free LPS but not by polysaccharide portion of LPS. ALF shows cell lysis activity against human polymorphonuclear leukocytes, mononuclear cells, and leukemia cells (K562), which are exposed with LPS (78).

Furthermore, ALF exhibits growth inhibitory activity against some, not all, strains of Gram-negative bacteria (77,82,83). Among the strains tested Re type strains, *Salmonella minnesota* R595 and *S. typhimurium* 1102, are most susceptible to the antibacterial action of ALF. Because ALF binds to lipid A portion of LPS, it seems likely that ALF neutralizes biologic activities of LPS. In fact, ALF suppresses the pyrogenicity of LPS in rabbits (92–94). Thus, it would be expected that ALF might be clinically useful to remove LPS and to suppress LPS pyrogenicity. As described earlier, ALF contains two positively charged regions and an amino-terminal hydrophobic region. If the two positively charged cluster regions form β-sheet and α-helix structure, respectively, the positive charges would cluster at the same side of the molecule. These two positively charged clusters might provide interaction site(s) with phosphate groups in the lipid A portion of LPS. Once ALF binds with LPS on cell membrane, the membrane structure seems to be perturbed by insertion of the hydrophobic amino-terminal region, including approximately up to 27th residues. The region appears to have a sufficient length to go across a lipid bilayer-like transmembrane α-helices of bacteriorhodopsin molecule (Fig. 15). The facts that ALF is localized in the L-granules and has strong antimicrobial activities do suggest that ALF is secreted during degranulation of the hemocyte induced by LPS and functions as antibactericidal substance following engulfment of the invaders by coagulation system (20).

Tachyplesins and Their Analogs. Tachyplesin I is a cationic peptide first isolated from acid extracts of Japanese horseshoe crab (*T. tridentatus*) hemocyte debris. This peptide inhibits the LPS-mediated activation of factor C in a similar manner to that of anti-LPS factor (74). Afterwards, two peptide analogs of tachyplesin I—tachyplesins II and III—have been isolated from hemocytes and two Southeast Asian horseshoe crabs, *T. gigas* and *C. rotundicauda* (75,79). Moreover, in the hemocyte debris of American *L. polyphemus*, two tachyplesin analogs, polyphemusins I and II, have also been found (75,90).

Tachyplesin is highly stable at low pH or high temperature, because the LPS-binding ability of tachyplesin is not affected even in 0.1% trifluoroacetic acid used for HPLC and by heat treatment in neutral pH buffer at 100°C for 30 minutes. This stability seems to be due to the

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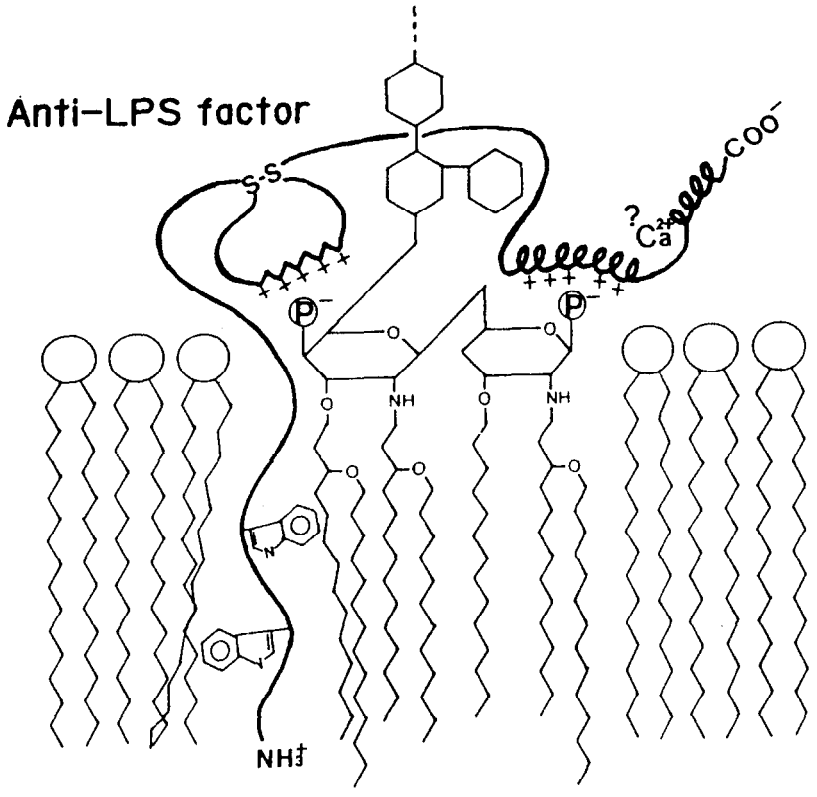


FIG. 15.

A speculative scheme of anti-LPS factor (ALF) which interacts with LPS at the positively charged regions and perturbs a cell membrane structure at the NH_2-terminal hydrophobic region.

rigid structure imposed by the two disulfide linkages. The primary structures of tachyplesin family are shown in Figure 16. They are highly basic polypeptides composed of 17 or 18 residues with arginine α-amide as the carboxyl-terminal end. Naturally occurring peptides containing arginine α-amide at the COOH termini have been reported in a scorpion polypeptide toxin and sarcotoxins from *Sarcophaga peregrina* (108). Tachyplesin shows a characteristic structure with three tandem repeats of a tetrapeptide sequence, i.e., hydrophobic amino acid-Cys-hydrophobic amino acid-Arg, indicating that its amphipathic nature is closely associated with biological activity (74,91). Figure 17 shows the conformational structure of tachyplesin I (80,85). The ^{1}H-N M R spectrum of tachyplesin I in aqueous solution could be completely assigned, and the secondary structure is substantiated by interpretations of the nuclear Overhauser effect, coupling constant, amide exchange rate, and temperature dependence of the amide chemical shift. Tachyplesin I takes on a fairly rigid conformation constrained by two disulfide bridges and adopts a conformation consisting of an antiparallel β-sheet (residues 3–8 and 11–16) connected by a β-turn (residues 8–11). In this planar conformation, five bulky hydrophobic side groups are localized in one side of the plane, and six cationic side groups are

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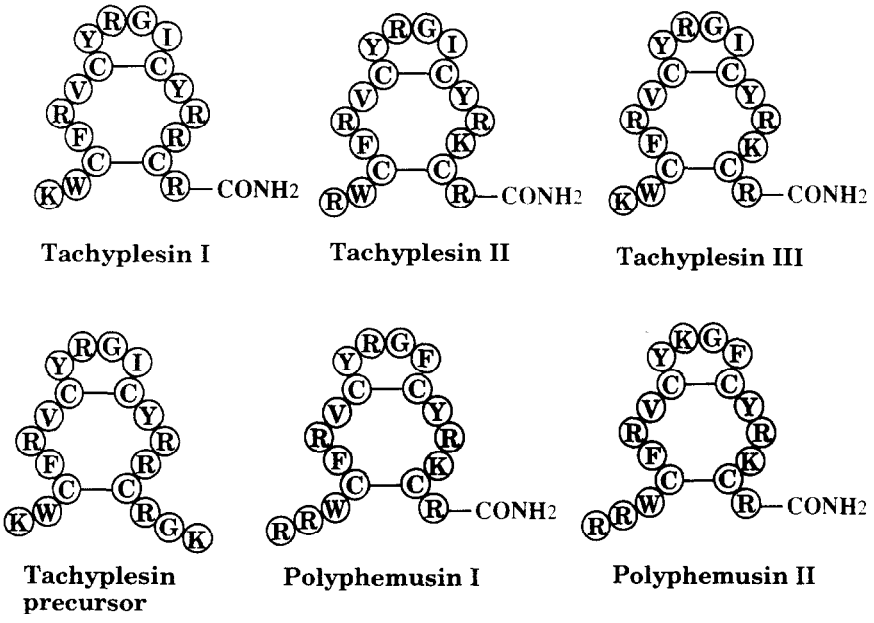


FIG. 16.

Primary structures of six kinds of tachyplesins and its analogs isolated from four species of horseshoe crabs. Tachyplesin III was isolated from *T. gigas*, I from *T. tridentatus*, *T. gigas* and *C. rotundicauda*, and II from *T. tridentatus* respectively. Polyphemusins I and II were isolated from *L. polyphemus*.

distributed at the "tail" part of the molecule (residues 1–5 and 14–17). This is presumed to be closely associated with the bactericidal activity (95).

As summarized in Table IV, tachyplesin displays potent antimicrobial activity against several strains of microorganisms (74,82). It is effective against not only rough and smooth strains

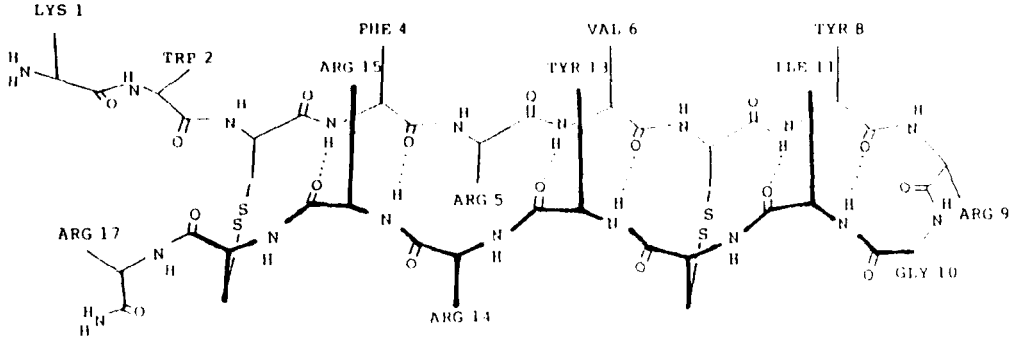


FIG. 17.

Schematic representation of the antiparallel β-pleated sheet structure of tachyplesin I.

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TABLE IV
Antimicrobial activity of native and synthetic tachyplesins and polyphemusins (75). Published by permission of the Japanese Biochemical Society, Tokyo. Original in Ref. 75.

	Minimal inhibitory concentration (μg/ml)						
	Tachyplesin I		Tachyplesin II		Polyphemusin I		Polyphemusin II
Strain	Native	Synthetic	Native	Synthetic	Native	Synthetic	Native
Gram (−)							
Salmonella typhimurium LT2	3.1	1.6–3.1	3.1	1.6–3.1	3.1	3.1–6.3	6.3
Salmonella typhimurium 1102	0.8–1.6	0.8–1.6	1.6	1.6–3.1	3.1	3.1	3.1
Escherichia coli K12	1.6–3.1	1.6–3.1	3.1	1.6–3.1	6.3	6.3	12.5
Salmonella minnesota 1114W	3.1	6.3	3.1	6.3	6.3	12.5	12.5
Salmonella minnesota R595	1.6	1.6	1.6	1.6	3.1	3.1	3.1
Pseudomonas aeruginosa	12.5	–	–	–	–	–	–
Gram (+)							
Staphylococcus aureus 209P	3.1	3.1	1.6–3.1	6.3	6.3	6.3	6.3
Staphylococcus aureus ATCC 25923	6.3	12.5	6.3	12.5	6.3	6.3	12.5
Bacillus subtilis	3.13	–	–	–	–	–	–
Fungus							
Candida albicans M9	3.1	3.1	3.1	3.1	6.3	6.3	6.3
Cryptococcus neoformans IMF40040	1.56	–	–	–	–	–	–

of Gram-negative but also Gram-positive cells, such as *Staphylococcus* species. In the presence of tachyplesin at 3.5 μg/ml, *Salmonella* strains irreversibly lose viability. This antimicrobial potency is comparable to that of anti-LPS factor previously described. Therefore, tachyplesin seems very likely to act as an antimicrobial peptide for the defense of horseshoe crabs against microbial infections (84,86).

Protein Precursors of Tachyplesins I and II. The cDNA encoding for tachyplesin precursors has been cloned and sequenced (Fig. 18). The tachyplesin precursors consist of 77 amino acids with 23 residues in a presegment, and there are two types of mRNAs corresponding to the isopeptides of tachyplesins I and II (81). Both precursors contain a putative signal peptide, a processing peptide sequence, and a carboxyl-terminal amidation signal "Gly-Lys-Arg" connected to the mature tachyplesin peptide. Moreover, an unusual acidic amino acid cluster with Asp-Glu-Asp-Glu-Asp-Asp-Asp-Glu-Glu-COOH is present in the carboxyl-terminal portions of both precursors. This acidic region might interact with a cationic part of the tachyplesin peptide to stabilize a conformational structure of the precursor for proteolytic degradation. The amino acid sequence of precursor molecule suggests that the mature tachyplesin might be generated through several steps of the processing. First, the amino-terminal signal sequence of the tachyplesin precursor is processed in a stepwise fashion by a signal peptidase followed by a dipeptidyl aminopeptidase, because the Glu-Ala-Glu-Ala sequence preceding the mature tachyplesin peptide is fit for the substrate specificity of a dipeptidyl aminopeptidase. Second, the cleavage of Arg-Asn bond at positions 20 and 21 is occurred by a processing enzyme that recognizes the dibasic sequence of Lys-Arg at positions 19 and 20. Third, release of Lys-19 and Arg-20 is taken place by a carboxypeptidase B-like enzyme, and finally an oxidative amidation is catalyzed by an enzyme recognizing the carboxyl-terminal glycine residue.

Northern blot analyses of total RNA prepared from various tissues indicate that the tachyplesin precursor is expressed exclusively in the hemocytes, in accordance with the high

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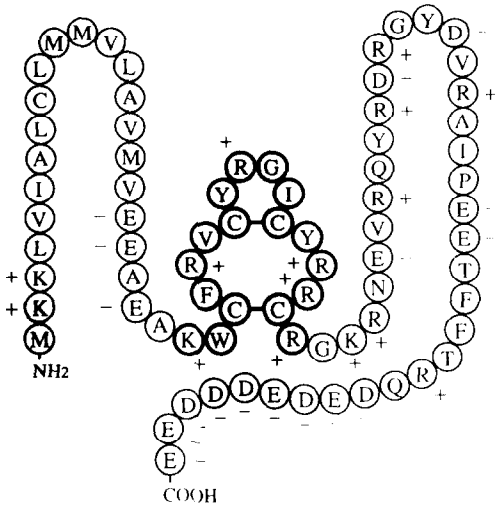


FIG. 18.

Amino acid sequence of tachyplesin I precursor. The residues circled thickly constitute a mature tachyplesin. The charged residues are indicated by + and −.

levels of tachyplesin peptides recovered therefrom (81). The mRNAs from heart and brain tissues also contain the transcript identical in size (0.7 kb) to that found in the hemocytes, but the signals are relatively weak. The amount of the tachyplesin precursor mRNA in other tissues, including those of the hepatopancreas, stomach, intestine, muscle, and coxal gland, is negligible. Although little is known about the hematopoietic tissue for production of hemocytes in horseshoe crab, our observations suggest that cardiac tissues may be the site of formation of circulating hemocytes. The subcellular localization of tachyplesin in the hemocytes proves to be in the S-granules, as determined immunohistochemically (20).

CONCLUSION

From all the results described previously, we speculate that bacterial endotoxin first contacts with an LPS receptor or LPS-binding proteins probably present in plasma membrane of the hemocyte and activates factor C located in the granules, and the resulting active factor C induces the activation of the intracellular clotting system (Fig. 19). The β-glucan-mediated pathway (67,68) also could not be disregarded for activation of this system. The clot generated during the activation encapsulates and immobilizes the Gram-negative bacteria, and the released peptide tachyplesin and anti-LPS factor act as the bactericidal substances. Therefore, this intracellular clotting system may have a crucial role in host defense against invading microorganisms.

Figure 20 shows an outline of the cellular and humoral defense systems in limulus. Although we have not mentioned about plasma components in this review, there are many humoral factors, such as proteinase inhibitors (87,96), α_2-macroglobulin (88,89), lectins (102), C-reactive protein (101), and polyphemin (4), all of which are important in antimicrobial defense (104). Furthermore, Liu and his colleagues (107) have also reported several endotoxin-

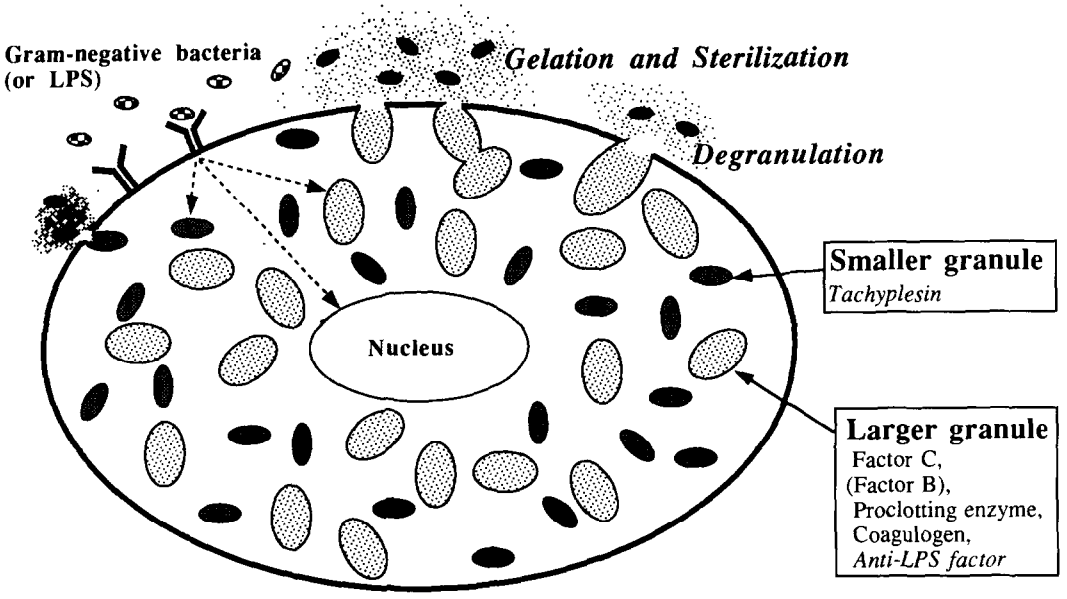


FIG. 19.

The hypothetical processes of coagulogen-based clotting system and containment of invading Gram-negative bacteria.

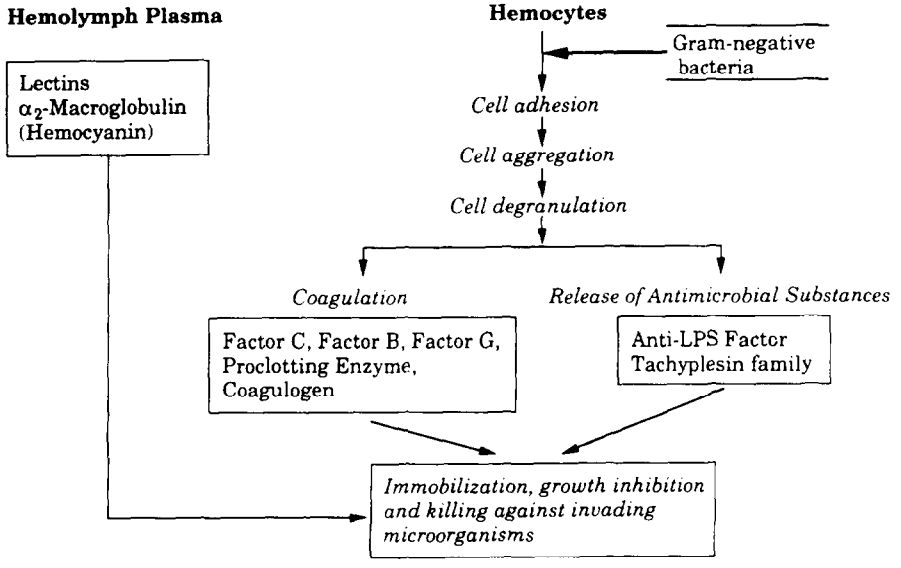


FIG. 20.

Cellular and humoral defense systems in *Limulus*.

binding proteins and a cell adhesion protein found in the limulus hemocytes (97-99,105,106). Although the exact functions of these substances are not known at present, they may act in concert with other components to serve as the biological defense of this animal (100,103). However, we have still less knowledge about cell biology and physiology of horseshoe crab hemocytes in comparison with mammalian blood cells.

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