

Horseshoe Crab Coagulation Factor B

A UNIQUE SERINE PROTEASE ZYMOGEN ACTIVATED BY CLEAVAGE OF AN ILE-ILE BOND*

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Horseshoe crab factor B is an intracellular serine protease zymogen involved in the bacterial endotoxin-responsive hemolymph coagulation cascade. cDNAs for factor B were isolated utilizing a polymerase chain reaction product using two primers derived from the partial amino acid sequence. The cloned cDNA of 1928 base pairs encoded 400 amino acid residues of factor B precursor. The first 23 amino acid residues constitute a presumed prepropeptide that may be processed by both a signal peptidase and a processing protease, similar to mammalian vitamin K-dependent protease precursors. The mature protein consists of 377 amino acids with a calculated molecular mass of 40,570 Da. The overall structure is highly homologous to that of limulus proclotting enzyme (35.9% identity), the substrate for active factor B in the cascade. Like the proclotting enzyme, mature factor B is composed of an amino-terminal "clip"-like domain and a carboxyl-terminal serine protease domain homologous to that of human plasma prekallikrein (36.5%). Internal sequences encode a unique activation peptide. Surprisingly, the cleavage sites of the zymogen factor B for activation by limulus active factor C were found to be an Arg-Ser and an Ile-Ile bond, the latter of which has not been found in any other protease zymogens. These cleavages result in the release of the activation peptide, which consists of 21 residues with a carboxyl-terminal isoleucine. These results indicate that the intracellular clotting system of the limulus hemocyte, like mammalian plasma clotting cascade, proceeds with the sequential activation of three serine protease zymogens: factor C, factor B, and proclotting enzyme.

A number of cascade systems have been described which serve to propagate and amplify various biological reactions. These can be generally divided into two groups: the serine protease zymogen and protein kinase cascades. Well known examples of the serine protease cascade systems include blood coagulation (1), fibrinolysis (2), complement systems (3), and prophenoloxidase activating system in insects (4). In the serine protease cascade system, the initiator is triggered by an appropriate stimulus, and the reaction is then propagated by successive activation accompanied usually by limited pro-

teolyses (1). Proteolysis proceeds irreversibly in the ordinary environment (5). This irreversibility dictates that the cascade components be tightly regulated; therefore, in addition to simple protease domains these zymogens also exhibit characteristic structural domains important for substrate specificity (6).

Among these cascades, the horseshoe crab (*limulus*) coagulation system is unique in that the reaction is initiated by bacterial endotoxin, lipopolysaccharide (7); moreover, the clotting factors are stored in granules in hemocytes (amebocytes) (8) and released into hemolymph by degranulation when the cells are activated (9). The clot formed through the activation of the cascade is effective for immobilizing invading microorganisms (10, 11), which are neutralized by antimicrobial substances secreted simultaneously from the cell (12-14). The clot formation is also triggered by (1,3)- β -D-glucan, which is present in the cell walls of yeast and other fungi (15, 16). This pathway is initiated by the activation of a serine protease zymogen, named factor G (17). Thus, the limulus clotting system is thought to be critical for both hemostasis and the microbiological defense of this animal.

In the past decade, we have investigated this cascade system and have found that it is composed of three serine protease zymogens and one clottable protein (coagulogen) (18). In the presence of lipopolysaccharide, a glycoprotein of 123 kDa, named factor C, is autocatalytically activated (18). The active factor C activates factor B to factor B⁺ by limited proteolysis (19). Then, the active factor B activates proclotting enzyme to clotting enzyme, which catalyzes the transformation of coagulogen to insoluble coagulum gel (20-22).

Zymogen factor C, which is sensitive to lipopolysaccharide, contains five complement-like ("sushi") domains, one epidermal growth factor-like domain, and one lectin-like domain (23, 24). The presence of these motifs may facilitate the molecule's participation in host defense system.

The zymogen factor B, on the other hand, is a glycoprotein of 64 kDa (19). Two zymogen forms are present in the purified preparation, one being a single-chain form and the other a nicked two-chain form composed of a light (L, 25 kDa)¹ and a heavy (H, 40 kDa) chain. Upon activation by factor C, the H chain of the two-chain form is proteolyzed, yielding an active serine protease with a shorter H chain (32 kDa). Interestingly, this activation is not catalyzed by trypsin, which activates most of serine protease zymogens found in these types of the cascades. As previously reported, autocatalytic cleavage site of zymogen factor C is an unusual Phe-Ile linkage (23, 24), suggesting that the cleavage site for activation of zymogen factor B might be trypsin-resistant. The present study was undertaken to elucidate the entire amino acid sequence of zymogen factor B and the mechanism underlying its activation.

¹ The abbreviations used are: L, light; H, heavy.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D14701.

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EXPERIMENTAL PROCEDURES

Materials—All DNA modifying enzymes were obtained from Nippon Gene Co., Toyama, Takara Shuzo Co., Kyoto, and Toyobo Co., LTD., Osaka, Japan. [α - ^{32} P]dCTP was from Amersham-Japan, Tokyo. Lysyl endopeptidase from *Achromobacter lyticus* was purchased from Wako Pure Chemical Industries, Ltd., Osaka. Phenyl-5PW RP and Chemcosorb 7 ODS-H columns were from Tosoh Corp., Tokyo, and Chemco Scientific Co., Ltd., Osaka, respectively. Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer. All other chemicals were of the highest quality commercially available.

Factor B was highly purified from the hemocyte lysate of *Tachyplesus tridentatus*, as described (19). λ gt11 cDNA libraries constructed from *T. tridentatus* hemocyte poly(A⁺) RNA were the same as that used previously (24).

Partial Amino Acid Sequence—The purified factor B was separated by SDS-polyacrylamide gel electrophoresis under reducing conditions (25), and the protein bands corresponding to the single, H, and L chains were electroeluted, respectively, to a Problot membraneTM (Applied Biosystems) and their sequences analyzed (26). The reduced and S-pyridylethylated L and H chains (27) were separated by reversed-phase high performance liquid chromatography on a column of Phenyl-5PW RP (4.6 $\times$ 75 mm). The isolated L and H chains were digested separately with lysyl endopeptidase, at a molar ratio of enzyme to substrate of 1:100. These digests were fractionated by reversed-phase high performance liquid chromatography on a column of Chemcosorb 7 ODS-H (2.1 $\times$ 150 mm). The isolated peptides were subjected to amino-terminal sequence analysis.

Polymerase Chain Reaction—Template DNA was extracted from the λ gt11 horseshoe crab hemocyte cDNA library (28). The primers used for the reaction, derived from amino acid sequences of N-D-N-E-C-T and (K)-N-F-G-I-G, were as follows: 5'-GCGAATTCA-AT/(C)GAT/(C)AAT/(C)GAA/(G)TGT/(C)AC-3' and 5'-GCGAATTCCA/(G/T)ATA/(C/G/T)CCA(G)AAA/(G)TTT/(C)TT-3'.

For the reaction, 1 μ g of the template, 150 pmol each of the oligonucleotide primers, and 2.5 units of Taq polymerase (Promega Co., Madison, WI) were used. The reactions for denaturation, annealing, and extension were carried out at 94 $^{\circ}$ C for 1 min, at 35 $^{\circ}$ C for 2 min, and at 60 $^{\circ}$ C for 2 min, respectively. The cycle was repeated 32 times. An aliquot of the product (1/50) was further amplified using the same protocol. The product was digested with *Eco*RI, purified with agarose gel electrophoresis, and subcloned into an *Eco*RI site of pUC118 (Takara Shuzo Co., Kyoto).

Screening—The above cloned polymerase chain reaction product (pFB15) used to screen the library was labeled with [α - ^{32} P]dCTP using a DNA labeling kit (Nippon gene Co.) as a probe. Five hundred thousand clones were screened at high stringency (28). Out of several positive plaques, two of the longest clones, λ FB51 and λ FB31, were sequenced after subcloning into an *Eco*RI site in the pUC118 vector.

Nucleotide Sequencing—Single stranded DNA templates were prepared from the plasmid vector pUC118 (29) and their nucleotide sequences were determined on an Applied Biosystems 370 A DNA Sequencer (30). Taq polymerase was used for extension reactions.

Northern Blotting—Total RNA was extracted from the hemocytes (31) and poly(A⁺) RNA was purified using oligotex dT-30 (32). Four μ g of poly(A⁺) RNA was separated on a 1.0% agarose gel and transferred to a GVHP membrane (Nippon Millipore Kogyo K.K., Yonezawa, Japan). Marker RNA (Life Technologies, Inc.) was electrophoresed simultaneously and stained with ethidium bromide. The filter was hybridized with the [^{32} P]pFB15 insert in 5 $\times$ SSC (1 $\times$ SSC = 15 mM sodium citrate and 150 mM NaCl, pH 7.0), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution = 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll), 50 mM sodium phosphate, pH 6.8, 50% formamide, 0.1 mg/ml yeast tRNA, and 0.5% sodium lauryl sulfate at 42 $^{\circ}$ C overnight and then washed in 0.1 $\times$ SSC containing 0.1% sodium lauryl sulfate at 65 $^{\circ}$ C (28). The washed filter was subjected to autoradiography.

Amino Acid and Sequence Analyses—Amino acid analysis was performed on a PICO-TAG system (Waters, Millipore Corp.) or a Hitachi L-8500 automatic analyzer. Amino acid sequence analyses were done using Applied Biosystems 477A or 473A Protein Sequencers (33).

Homology Search—The amino acid sequence of factor B was compared with all entries in the NBRF Protein Sequence Database (release 23.0, March, 1992) with GENETYX system (Software Development Co., LTD. Tokyo) based on the algorithm of the computer program FASTP (34).

RESULTS

The zymogen factor B purified from the hemocyte lysate contained both a two-chain form, the major species, and a single-chain form (19). Each chain was separated by SDS-polyacrylamide gel electrophoresis and its amino-terminal sequence was analyzed. The single-chain form consisted of two different amino-terminal sequences; VGVLFPPKT and VLFPPKT, suggesting that the first 2 residues had been removed during the preparation of zymogen factor B. Since the sequence of the L chain was identical to these sequences, its location is amino-terminal relative to the H chain on the intact molecule, similar to other horseshoe crab coagulation factors, factor C (24), and proclotting enzyme (33). The amino-terminal sequence of the isolated H chains from the zymogen and the active form were SGSPILPPI and IAGGVEAKI, the former corresponding to the sequences of H chain containing the activation peptide (described later) and the latter to that of serine protease domain. The S-alkylated L and H chains were digested with lysyl endopeptidase and the purified peptides derived from both chains were sequenced (Fig. 3, *underlined*).

The two longest positive clones from the screening of the λ gt11 hemocyte cDNA library, named λ FB51 and λ FB31, were chosen and subjected to sequence analysis (Fig. 1). Northern blot analysis of the hemocyte poly(A⁺) RNA with the polymerase chain reaction product (pFB15) exhibited a single band of about 2.1 kilobases, indicating that the clone, λ FB51, was close to the full-length clone (Fig. 2).

λ FB51 was composed of 1928 base pairs, whose sequence completely agreed with that of λ FB31 (Fig. 3). Because termination codons were present in all three reading frames of its 5'-region, the initiation codon ATG could be assigned to begin with nucleotide 143. After the 5'-noncoding region, the initiation codon was followed by a 1200-base pair open reading frame, a TGA termination codon, a 586-base pair 3'-noncoding region, and a poly(A) tail. A poly(A) signal, ATTAAA, was found 23 residues upstream of the poly(A) tail.

The deduced amino acid sequence from the first ATG initiation codon included all the partial sequences of factor B, which comprised 54% of the mature protein (Fig. 3, *underlined*). A 23-amino acid amino-terminal sequence, presumably

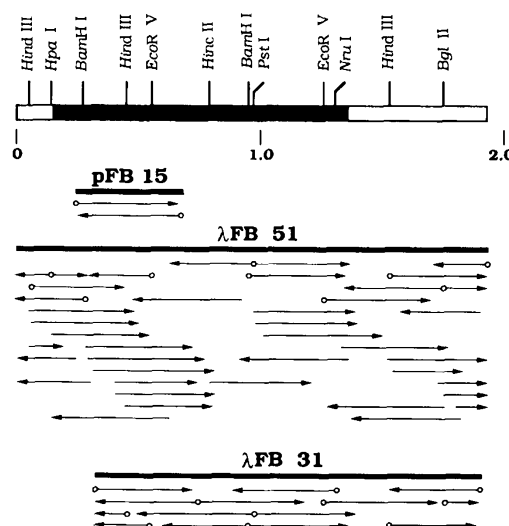


FIG. 1. Restriction map and sequence strategy. A black box in the restriction map represents an open reading frame. The nucleotide sequence analyses were carried out with an Applied Biosystems 370A DNA Sequencer in the directions and length indicated by arrows, after being subcloned into pUC118 vector.

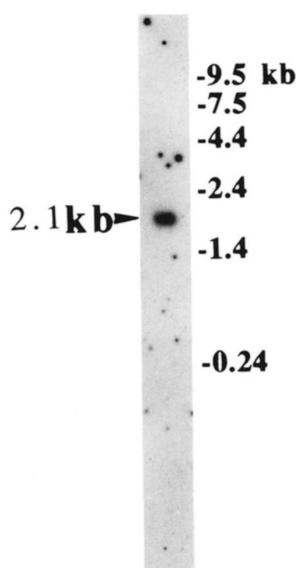


FIG. 2. **Northern blot analysis.** Four μ g of poly(A⁺) RNA from the hemocytes was separated by 1.0% agarose gel electrophoresis, transferred to a GVHP membrane, and hybridized with the pFB15 insert. See "Experimental Procedures" for details. kb, kilobase.

corresponding to the prepropeptide, preceded the mature protein consisting of 377 amino acids with a calculated molecular mass of 40,570 Da. Three potential glycosylation sites for *N*-linked carbohydrate chain (Asn-X-Ser/Thr) were present in the mature protein sequence (Asn-117, Asn-228, and Asn-329).

Because the amino-terminal sequences of the H chains of the two-chain zymogen form and the active form were found to begin with Ser-104 and Ile-125, respectively, an activation peptide with 21 amino acids from Ser-104 to Ile-124 was presumably liberated during zymogen activation. In fact, the amino acid composition of the activation peptide released after activation of zymogen factor B with factor C was in good agreement with that predicted from the cDNA sequence (data not shown). The peptides also contained hexosamines, probably derived from carbohydrate moiety linked with Asn-117 and Ser/Thr residues in the activation peptide. Among the three protease zymogens constituting the limulus clotting cascade, only factor B releases such an activation peptide from the parental molecule during its activation (19).

The cleavage site directly associated with its activation by factor C was presumed to be the peptide bond between Ile-124 and Ile-125. This result explains why zymogen factor B, like factor C, is not susceptible to trypsin (23, 35).

DISCUSSION

The results of our experiments demonstrate that factor B is encoded by a single open reading frame of 1200 nucleotides consisting of a 23-amino acid prepropeptide followed by a 377-amino acid mature protein. The entire amino acid sequence of the mature protein shows the most homology with that of proclotting enzyme previously cloned in our laboratory, as shown in Fig. 4 (33). The sequences of the L chain portion share a significant similarity (25.0%) as do the H chains corresponding to serine protease domains (42.4%). The overall amino acid sequence identity is 35.9%, with a gap inserted in the region of the activation peptide derived from factor B. This fact strongly suggests that these two proteases have evolved from a single gene by duplication.

The amino-terminal portion of the factor B-derived L chain

has 6 cysteines which correspond to the positions of those in proclotting enzyme. Since these disulfide locations have been determined in proclotting enzyme (33), the cysteine residues found in this region of factor B are probably bridged in the same topology with three disulfide bonds as follows; Cys¹⁴-Cys⁵⁶, Cys²⁴-Cys⁴⁶, and Cys³⁰-Cys⁵⁷ (Fig. 5). This structure forms a discrete domain, called "clip"-like domain, named based on the disulfide bonds (33).

Recently, Dr. A. Day informed us that the L chain of proclotting enzyme has a significant sequence similarity with a tentative *Drosophila* serine protease precursor, named easter.² The juxtaposition of these sequences, as shown in Fig. 5, demonstrates that the 6 cysteine residues are similarly aligned. Easter is an essential serine protease zymogen for pattern formation in normal embryonic development (36). Although the significance is still unknown, these results suggest that the clip-like domain found in the horseshoe crab clotting factors is a common structural element in the serine protease zymogens of invertebrates and that a similar protease cascade might be present in other species.

The 253-amino acid factor B-derived H chain contains a typical trypsin-type serine protease domain with active site triads, histidine, aspartic acid, and serine and a conserved primary substrate binding site, Asp³¹⁵ (Ser¹⁸⁹ in chymotrypsin numbering). The molecule exhibiting the most homology was the limulus proclotting enzyme (42.4%), and among other known mammalian serine proteases, human plasma kallikrein exhibits the highest similarity (36.5%) (37). This is not surprising, since the closest protease to proclotting enzyme has been found in the protease domain of human factor XI (33), and plasma prekallikrein has a well known sequence similarity to factor XI (38–40). These results indicate that the serine protease domains of factor B/proclotting enzyme of horseshoe crabs are related to the factor XI/prekallikrein family in humans, and may have evolved from a common origin.

Out of 6 cysteines in the factor B-derived H chain, 5 cysteines can be easily assigned from the structure of proclotting enzyme to be involved in three disulfide bonds (33): Cys²⁸⁵-Cys³⁰⁶, Cys³¹⁷-Cys³⁴⁵, and Cys²³⁷-Cys⁸⁹ (in the L chain). The remaining cysteine, Cys¹⁵⁴, is present at the position of the first cysteine located in the so-called "histidine loop" of serine proteases. In the position of its expected counterpart cysteine at residue 170, however, is an alanine. Other such serine proteases without the histidine loop have been previously described (41). However, in these proteins, not one but both cysteines have been substituted. Consequently, this Cys¹⁵⁴ should exist as a free cysteine residue or a mixed disulfide linked with a small compound, such as glutathione or cysteine.

The most striking finding is that the amino-terminal isoleucine (Ile¹²⁵) of the H chain in the activated factor B was preceded by Ile¹²⁴ in the zymogen form, while the other proteolytic cleavage site not involved in the activation is an Arg¹⁰³-Ser¹⁰⁴ bond. The only activator for zymogen factor B known thus far is factor C, which must cleave the peptide bond between Ile¹²⁴ and Ile¹²⁵. Although factor C contains a conserved Asp¹⁸⁹ (chymotrypsin numbering), like trypsin-type serine proteases, the autocatalytic activation site has been identified to be Phe-Ile bond (23, 24). Thus, factor C has endogenous chymotrypsin-like activity, in addition to trypsin-like activity. Since the sequences around this activation site in zymogen factor B have been confirmed in three independent cDNA clones, it is unlikely to be a cloning artifact. Furthermore, the existence of this sequence is consistent with the fact that factor B is activated neither by trypsin nor α -

² A. Day, personal communication.

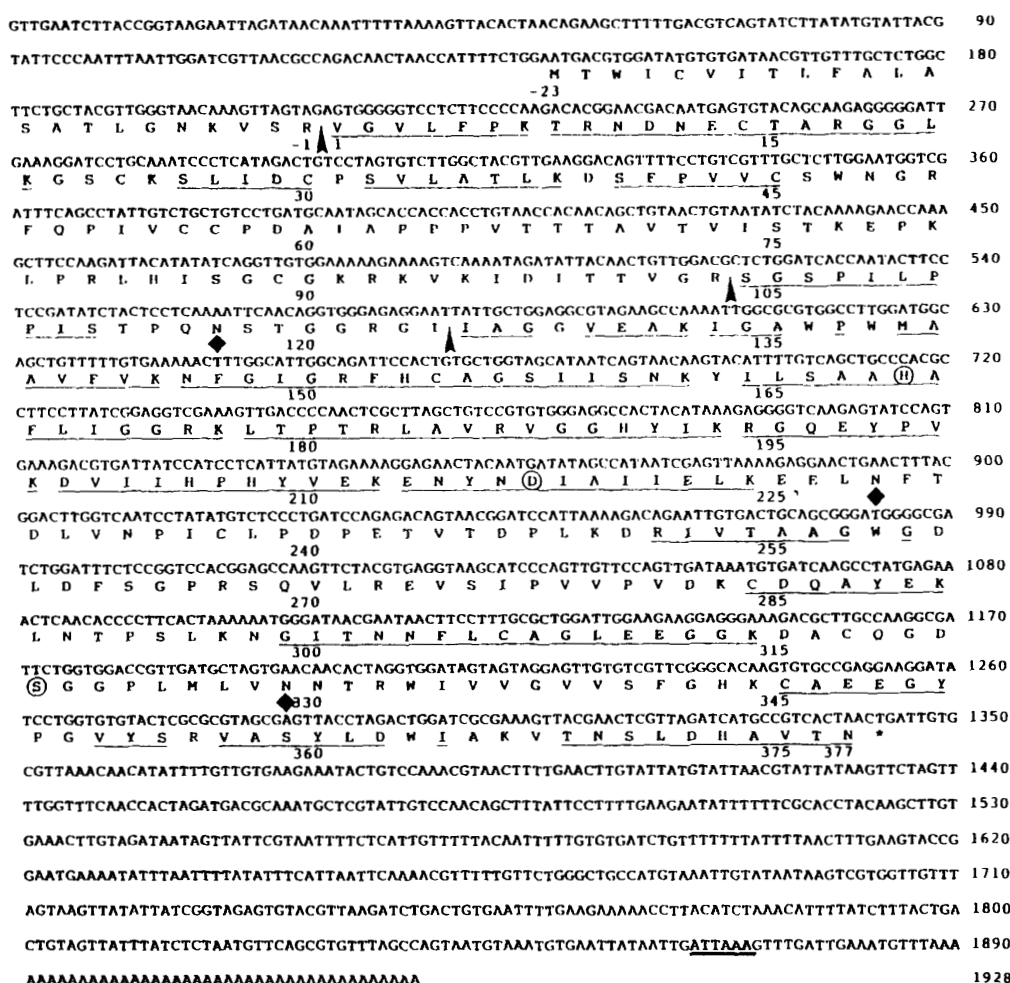


FIG. 3. Nucleotide and deduced amino acid sequences of factor B. Nucleotide (*upper*) and deduced amino acid sequences (*lower*) are shown. The amino acid sequences are numbered from the amino terminus of the mature protein. The *underlined* amino acid residues were confirmed by protein sequencing. The cleavage sites accompanied with 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*.

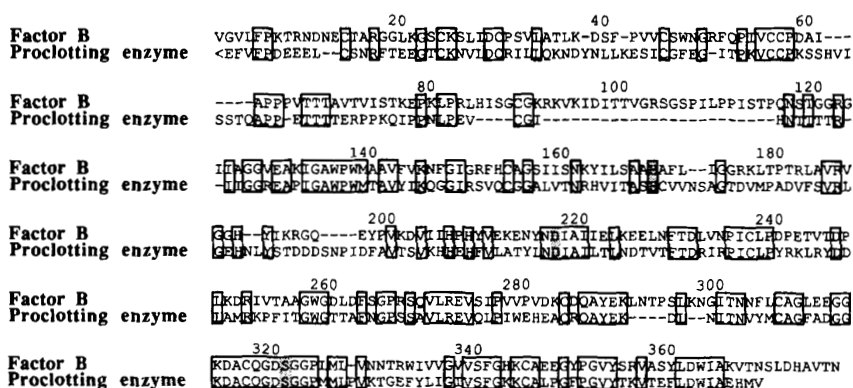


FIG. 4. Alignment of sequences of horseshoe crab factor B and proclotting enzyme (33). Identical residues are boxed. The amino acid residues suspected to form the catalytic triad of the serine protease domain are shadowed. The numbering is that of factor B.

chymotrypsin.³ Although the *Drosophila snake* gene product has been speculated to contain a Leu-Ile activation site based on homology data (42), only limulus factor B and C have been proven to contain such an activation site. Probably, this type of cleavage site for zymogen activation might prevent an

unexpected positive feedback mediated by trypsin-like serine proteases in horseshoe crab hemocytes, such as clotting enzyme (20) and factor G (15) or factor B itself. Moreover, this unique activation mechanism as well as that of factor C (23) might be involved in the regulation of limulus clotting cascade.

The zymogen factor B contains a prepro sequence consisting of 23 amino acid residues at its amino terminus. The

³ T. Muta, T. Oda, and S. Iwanaga, unpublished results.

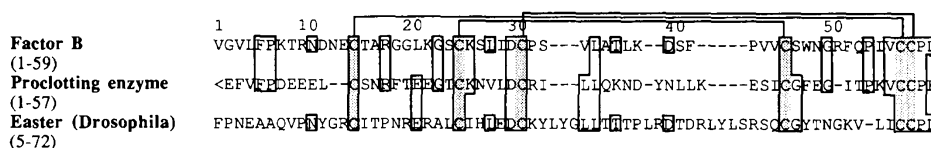


FIG. 5. Alignment of sequences of clip-like domains found in horseshoe crab factor B, proclotting enzyme (33), and *Drosophila easter* (36). Identical residues are boxed. Cysteines are shadowed. Disulfide bonds indicated by solid lines are determined in proclotting enzyme (33). The numbering is that of factor B.

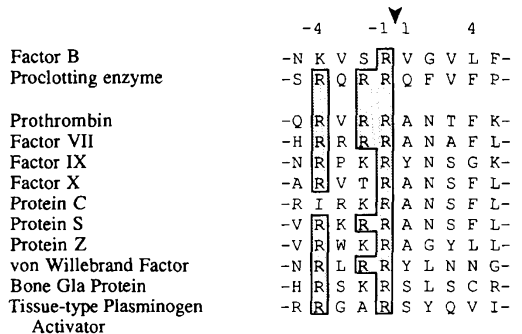


FIG. 6. Alignments of sequences around the tentative processing site of factor B and other proteins. Identical residues are boxed. The amino acids are numbered with mature amino terminus as +1. Sequences are from the review article (41).

amino-terminal region of the sequence is highly hydrophobic and contains a typical signal sequence. According to the algorithm of von Heijne (43), a signal peptidase should cleave between residue -9 and -8 or -6 and -5. The resulting carboxyl-terminal region shows an interesting sequence. The corresponding amino acid residues from -4 to -1 are -Lys-Val-Ser-Arg. This sequence is unlikely to be cleaved by a signal peptidase, thus suggesting a propeptide region of the immature factor B. Some of the well known examples of such propeptides are the vitamin K-dependent proteases in mammalian clotting factors, whose propeptides show a conserved sequence, -Arg-X-Arg-Arg (amino acid -4 to -1), as shown in Fig. 6. Although the propeptide sequence of factor B is not a typical one, basic residues at P₄ and P₁ have been shown to be very important for recognition by the processing enzyme found in mammals. Likewise, proclotting enzyme shows the typical sequence, -Arg-Gln-Arg-Arg, and factor G also contains a similar prosequence, although its molecular organization differs from proclotting enzyme and factor B.³

The present work completes the cDNA cloning for the essential components of the endotoxin-sensitive coagulation cascade: factor C (24), factor B (this work), proclotting enzyme (33), and coagulogen (22). This coagulation cascade is being widely utilized as a sensitive biological test for bacterial endotoxins. Although several regulatory proteins might be associated with the cascade, these cDNAs may be useful to reconstitute this reaction by recombinant molecules.

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