

Factor B Is the Second Lipopolysaccharide-binding Protease Zymogen in the Horseshoe Crab Coagulation Cascade*

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Background: It is not known whether LPS is required for the activation of coagulation factor B.

Results: Factor B is an LPS-binding zymogen activated by α -factor C in an LPS-dependent manner.

Conclusion: The clip domain of factor B has an important role in localizing factor B to LPS.

Significance: Horseshoe crab coagulation is an ideal model for understanding proteolytic cascades.

Factor B is a serine-protease zymogen in the horseshoe crab coagulation cascade, and it is the primary substrate for activated factor C, the LPS-responsive initiator of the cascade. Factor C is autocatalytically activated to α -factor C on LPS and is artificially converted to β -factor C, another activated form, by chymotrypsin. It is not known, however, whether LPS is required for the activation of factor B. Here we found that wild-type factor B expressed in HEK293S cells is activated by α -factor C, but not by β -factor C, in an LPS-dependent manner and that β -factor C loses the LPS binding activity of factor C through additional cleavage by chymotrypsin within the N-terminal LPS-binding region. Surface plasmon resonance and quartz crystal microbalance analyses revealed that wild-type factor B binds to LPS with high affinity comparable with that of factor C, demonstrating that factor B is the second LPS-binding zymogen in the cascade. An LPS-binding site of wild-type factor B was found in the N-terminal clip domain, and the activation rate of a clip domain deletion mutant was considerably slower than that of wild-type factor B. Moreover, in the presence of LPS, Triton X-100 inhibited the activation of wild-type factor B by α -factor C. We conclude that the clip domain of factor B has an important role in localizing factor B to the surface of Gram-negative bacteria or LPS released from bacteria to initiate effective proteolytic activation by α -factor C.

zymogens—factor C, factor G, factor B, and the proclotting enzyme—plays a pivotal role in hemolymph coagulation (3): factor C triggers the hemolymph coagulation cascade in response to stimulation with bacterial LPS, which activates factor B to convert the proclotting enzyme to the clotting enzyme, thereby promoting transformation of the clottable protein coagulogen to coagulin. On the other hand, factor G directly activates the proclotting enzyme in response to stimulation with β -1,3-D-glucans (3). Factor C contains an LPS-binding site located in the N-terminal Cys-rich region, and a tripeptide sequence of -Arg³⁶-Trp³⁷-Arg³⁸—in this region is essential for LPS recognition (4) (see Fig. 1). In addition, the N-terminal Arg residue of factor C and the distance between the N terminus and the tripeptide are absolutely required for specific protein-protein interactions to form multiple oligomers of factor C on the surface of LPS, leading to autocatalytic activation (5).

The protease zymogens in the horseshoe crab coagulation cascade are members of the trypsin family. Trypsinogen is activated by cleavage of the -Arg¹⁵-Ile¹⁶- bond in chymotrypsinogen numbering by enteropeptidase, following insertion of the newly appearing N-terminal Ile¹⁶ into the Ile¹⁶ cleft to form a salt bridge between the α -amino group of Ile¹⁶ and the side chain of Asp¹⁹⁴, leading to conformational changes in the substrate binding site and the oxyanion hole required for enzymatic catalysis (6). Interestingly, factor C is converted to α -factor C in the presence of LPS, and autocatalytic cleavage occurs within the -Phe⁷³⁷-Ile⁷³⁸- bond (see Fig. 1). On the other hand, this peptide bond is artificially cleaved by chymotrypsin, which produces another activated form, β -factor C (5, 7). Wild-type factor C expressed in an HEK293 mutant cell line lacking N-acetylglucosaminyltransferase (GnTI),³ containing restricted and homogeneous N-glycans of Man₅GlcNAc₂ (8), exhibits LPS-induced activity equivalent to that of native factor C (5). Both activated forms of factor C exhibit amidase activity against a synthetic peptide substrate, butoxycarbonyl (Boc)-Val-Pro-Arg-4-methylcoumaryl-7-amide (MCA) (5).

Several protease zymogens are involved in proteolytic cascades triggered on the surfaces of biologic substances derived from host tissues, cells, or invading pathogens to amplify and propagate various biologic reactions, such as blood coagulation and complement systems in mammals (1, 2). In horseshoe crabs, a proteolytic cascade comprising four serine-protease

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³ The abbreviations used are: GnTI, N-acetylglucosaminyltransferase I; Boc, butoxycarbonyl; MCA, 4-methylcoumaryl-7-amide; pNA, p-nitroanilide; SAM, self-assembled monolayers.

Activation of Coagulation Factor B on Lipopolysaccharide

Factor B is the primary substrate of α -factor C, and it is proteolytically activated through cleavage of the -Ile¹²⁴-Ile¹²⁵-bond (9) (see Fig. 1). Factor B contains a unique motif at the N-terminal region, named the clip domain, which was first identified in the proclotting enzyme of horseshoe crabs (10–12). Later, several clip domains were reported in serine proteases and protease homologs in arthropods, such as *Drosophila* dorso-ventral polarity, and in innate immune systems, including the activation of prophenoloxidase and production of antimicrobial peptides (13–16). The functions of these clip domains, however, are not well understood. We hypothesize that the clip domain of factor B is involved in a specific interaction between factor B and α -factor C on LPS, leading to the proteolytic activation of factor B. It is not clear, however, whether LPS is required for the activation of factor B. To elucidate the molecular mechanism underlying factor B activation, we prepared several recombinants of factor B expressed in the HEK293 mutant cell line, the same as that used for studies of factor C (5). Here we show that wild-type factor B is activated by α -factor C, but not by β -factor C, in an LPS-dependent manner and that factor B is the second LPS-binding protease zymogen in the cascade. We also report that the LPS-binding site of factor B is localized in the clip domain and that the clip domain has an important role in localizing factor B to the surface of LPS, leading to its efficient activation by α -factor C.

Experimental Procedures

Materials—The HEK293S GnTI[−] cell line was obtained from ATCC. LPS derived from *Salmonella minnesota* R595 (Re) was purchased from List Biological Laboratories, Inc. (Campbell, CA) and used for the factor C or factor B activation assays and as free LPS to evaluate binding to LPS-immobilized Sepharose. Biotinylated LPS prepared from *Escherichia coli* O111 was purchased from InvivoGen (San Diego, CA) and used for the preparation of LPS-immobilized Sepharose and surface plasmon resonance and quartz crystal microbalance analyses. Boc-Val-Pro-Arg-*p*-nitroanilide (pNA) was provided by Seikagaku Corporation (Tokyo, Japan). Boc-Leu-Thr-Arg-MCA and succinyl-Ala-Ala-Pro-Phe-MCA were purchased from Peptide Institute, Inc. (Osaka, Japan).

Cloning and Mutagenesis—The full-length DNA fragment of factor C (17), factor B (9), or the clip domain of factor B (9), derived from the horseshoe crab *Tachypleus tridentatus*, was subcloned into vector pCA7 (18), and a sequence derived from pHlsec (19) was used as a secretion signal sequence. An insert containing a six-histidine tag and a cleavage site of factor Xa was added to the N-terminal end of factor C by site-directed mutagenesis using inverse PCR. A GST tag was inserted into the N terminus of the clip domain.

Expression of Recombinant Proteins—Recombinant proteins were expressed as described previously (5). In brief, recombinant proteins were expressed in HEK293S GnTI[−] cells and secreted into the culture medium. HEK293S GnTI[−] cells were maintained in DMEM supplemented with 1% glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum at 37 °C under 5% CO₂. DMEM supplemented with plasmids containing the inserts of the recombinant proteins (1.8 μ g/ml), polyethylenimine (2.7 μ g/ml), 1% glutamine, 1% penicillin-strepto-

mycin, and 2% fetal bovine serum was used to transfect HEK293S GnTI[−] cells at 80–90% confluence. Culture media were collected 5 days after transfection and centrifuged at 6000 rpm for 30 min.

Purification of Wild-type Factor C—Collected culture media containing wild-type factor C with the histidine tag and the cleavage site of factor Xa were mixed with 0.1 volumes of 0.5 M NaH₂PO₄-NaOH, pH 8.0, containing 1.5 M NaCl and 0.1 M imidazole and applied to a nickel-nitrilotriacetic acid-agarose column (1.0 $\times$ 5.0 cm). The column was washed with 50 mM NaH₂PO₄-NaOH, pH 8.0, containing 150 mM NaCl and 10 mM imidazole, and wild-type factor C was eluted with 50 mM NaH₂PO₄-NaOH, pH 8.0, containing 150 mM NaCl and 20–200 mM imidazole. The eluted fraction buffer was exchanged with 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 10 mM CaCl₂, after which factor Xa was added and incubated for 16 h at 37 °C. Factor Xa was removed by applying to a benzamidine-Sepharose column (0.25 $\times$ 1.5 cm), and the flow-through fraction was applied to a nickel-nitrilotriacetic acid-agarose column (0.25 $\times$ 1.5 cm) to remove the free histidine tag. The concentration of wild-type factor C was determined using the extinction coefficient of 1% solution at A_{280 nm} of 21.3 (20).

Purification of Wild-type Factor B, the Clip Domain Deletion Mutant (Δ Clip-factor B), and the GST-conjugated Clip Domain (GST-clip)—Culture medium containing wild-type factor B or Δ clip-factor B was 5-fold diluted with 50 mM NaH₂PO₄-NaOH, pH 6.8, and applied to an SP-Sepharose column (1.0 $\times$ 10 cm). The column was washed with 50 mM NaH₂PO₄-NaOH, pH 6.8, and the recombinant protein was eluted with 50 mM NaH₂PO₄-NaOH, pH 6.8, containing 50–500 mM NaCl. After fractions containing the recombinant protein were pooled, the buffer was exchanged for 20 mM Tris-HCl, pH 8.0, by ultrafiltration and applied to a DEAE-Sepharose column (1.0 $\times$ 1.0 cm) for further purification. The recombinant protein was eluted with 20 mM Tris-HCl, pH 8.0, containing 10–200 mM NaCl, and concentrated by ultrafiltration. Culture medium containing the GST-clip domain was mixed with 0.1 volumes of 0.5 M NaH₂PO₄-NaOH, pH 7.6, containing 1.5 M NaCl and 10 mM dithiothreitol and applied to a glutathione-Sepharose column (1.0 $\times$ 1.0 cm). The column was washed with 50 mM NaH₂PO₄-NaOH, pH 7.6, containing 150 mM NaCl, and the GST-clip domain was eluted with 50 mM NaH₂PO₄-NaOH, pH 7.6, containing 150 mM NaCl and 20 mM glutathione. The concentrations of these three recombinant proteins were determined by the Micro BCATM protein assay kit (Thermo Scientific).

Activation of Wild-type Factor C and Factor B—Wild-type factor C (170 nM, 20 μ g/ml) was activated by LPS (360 nM, 6 μ g/ml) or chymotrypsin (200 nM, 5 μ g/ml) for 10 or 30 min, respectively, at 37 °C; 0.1 mM tosyl-L-phenylalanine chloromethyl ketone was added to stop the reaction with chymotrypsin. The resulting α -factor C or β -factor C was diluted with 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 1.5 μ M BSA (100 μ g/ml) to prepare several concentrations. The amidase activity of α -factor C or β -factor C in the presence or absence of LPS was assayed with 0.4 mM Boc-Val-Pro-Arg-pNA. Wild-type factor B (120 nM, 5.0 μ g/ml) was activated by α -factor C or β -factor C in the presence or absence of LPS for 5 min at 37 °C.

The amidase activity of activated factor B was assayed with 0.4 mM Boc-Leu-Thr-Arg-MCA, as described previously (5). Molar concentrations of pNA and 7-amino-4-methylcoumarin released were measured, respectively, by absorbance at 405 nm and fluorometrically with excitation at 380 nm and emission at 440 nm.

Protein Sequence Analysis— β -Factor C was subjected to SDS-PAGE, followed by electroblotting to a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue R-250, and the N-terminal sequence of the stained band was analyzed by Edman degradation (Genostaff, Tokyo, Japan).

Antibody Preparation—To prepare the antibodies against factor B and the L chain of factor C, synthesized peptide antigens were used. Peptides of FPKTRNDNEC and Cys-GDLDFS-GPRSQ for factor B or those of Cys-GKYYRDDSRDDYVQ and Cys-ADDSRTERRWVLEG for the L chain of factor C were used to immunize rabbits (Scrum, Tokyo, Japan).

Western Blotting—Samples were subjected to SDS-PAGE in 10% or 12% slab gel and transferred to a PVDF membrane. After blocking with 5% skim milk, the membrane was incubated with polyclonal antibody against factor B or factor C, monoclonal antibody 2C12 against factor C (21), or anti-GST tag HRP-DirecT antibody (MBL, Nagoya, Japan), after which it was incubated with the secondary antibody (HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG; Bio-Rad), followed by development with WesternBright Quantum (Advansta, Menlo Park, CA). Precision Plus Protein Dual Color standards (Bio-Rad) were used to determine apparent molecular mass.

Binding Assays to LPS-immobilized Sepharose—Streptavidin-immobilized Sepharose (Sigma-Aldrich) was incubated with or without biotinylated LPS for 1 h at 4 °C in 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 1.5 μ M BSA. Sepharose obtained by centrifugation at 2000 rpm for 3 min was washed with 1.0 ml of 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. The resulting LPS-immobilized Sepharose or control Sepharose was incubated with samples in 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 1.5 μ M BSA in the presence or absence of free LPS for 1 h at 4 °C and washed three times. Sepharose reacted with the recombinant proteins was obtained by centrifugation and subjected to Western blotting.

LPS Binding Assays by Surface Plasmon Resonance Analysis and by a Quartz Crystal Microbalance—Surface plasmon resonance analysis was performed as described previously (5). Biotinylated LPS (2.3 μ M in 10 mM HEPES-NaOH, pH 7.4, containing 150 mM NaCl) was immobilized on a sensor chip SA of a BIAcore X system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's specifications. Wild-type factor B was injected at a flow rate of 30 μ l/min in the running buffer, 10 mM HEPES-NaOH, pH 7.4, containing 150 mM NaCl. The change in the mass concentration on the sensor chip was monitored as a resonance signal using the program supplied by the manufacturer. Sensorgrams of the interactions obtained using the various concentrations of wild-type factor B (10, 20, 30, 40, and 50 nM) were analyzed by the BIAevaluation program, version 3.0 (GE Healthcare).

The interaction of wild-type factor B with LPS was also examined using the 27-MHz quartz crystal microbalance, Affinix Q (Initium Co., Tokyo, Japan). Biotinylated LPS was immo-

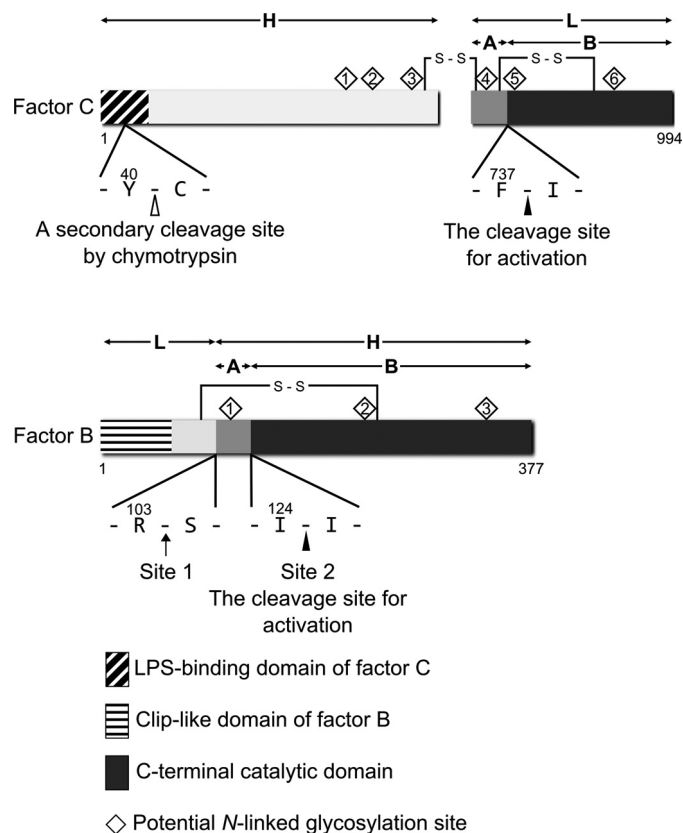


FIGURE 1. Schematic domain structures of factor C and factor B.

bilized onto a 27-MHz electrode by Biotin-SAM formation reagent (Dojindo Laboratories, Mashiki-machi, Kumamoto, Japan). The biotin-SAM was prepared by immersing a clean electrode in 0.1 mM Biotin-SAM solution dissolved in ethanol at room temperature for 2 h. The Biotin-SAM-coated electrode was rinsed with ethanol and washed with water. Then avidin (20 μ g/ml) was immobilized on the Biotin-SAM-coated electrode in 10 mM HEPES-NaOH, pH 7.4, containing 150 mM NaCl. After washing the avidin-immobilized electrode with water, biotinylated LPS was immobilized on the electrode in the same buffer. The LPS-immobilized electrode was washed with water several times for removal of the excess LPS and then soaked in the same buffer. Wild-type factor B was added to the solution and monitored continuously for frequency changes at 25 °C. The frequency changes in response to the various concentrations of these proteins were assessed, and the dissociation constant was determined according to the published method (22).

Results

Wild-type Factor B Is Activated by α -Factor C, but Not by β -Factor C—Native factor B purified from horseshoe crab hemocytes was purified as a two-chain form of the zymogen consisting of L and H chains through proteolytic cleavage of the -Arg¹⁰³-Ser¹⁰⁴- bond (site 1) by unknown protease(s) (Fig. 1) (23). In this study, wild-type factor B expressed in HEK293S GnTI⁻ cells contained not only the two-chain form but also a single-chain form of the zymogen indicated by Factor B in Fig. 2A. Wild-type factor B was activated by α -factor C in the pres-

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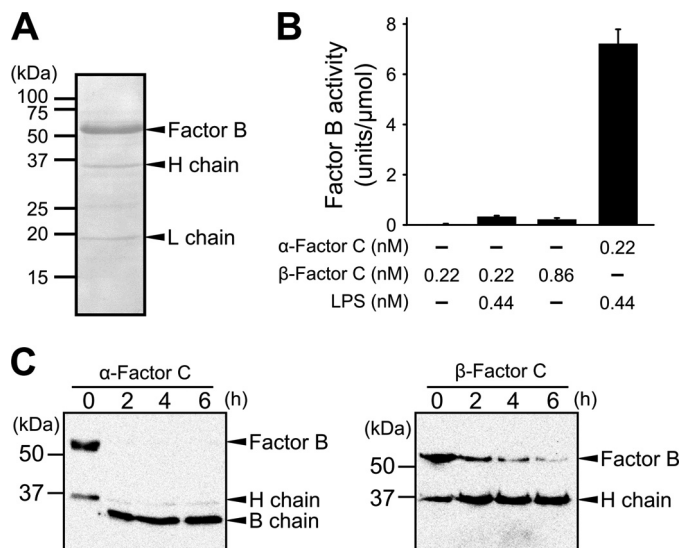


FIGURE 2. Activation of wild-type factor B by activated factor C. A, wild-type factor B was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. B, wild-type factor B (120 nM) was incubated with α -factor C (0.22 nM) or β -factor C (0.22 or 0.86 nM) for 5 min. Wild-type factor B was also incubated with β -factor C for 5 min in the presence of LPS (0.44 nM). The resulting factor B activity was assayed with Boc-Leu-Thr-Arg-MCA. Amidase activity was defined as μ mol of the digested substrate per min, and the specific activity was expressed as units per μ mol of factor B. The data are the means $\pm$ S.D. of three independent experiments. C, wild-type factor B (120 nM) was incubated with α -factor C (0.22 nM) or β -factor C (0.22 nM) in the presence of LPS (0.44 nM) for the indicated durations and subjected to Western blotting with anti-factor B polyclonal antibody.

ence of LPS (Fig. 2B). To investigate whether LPS is required for the activation of wild-type factor B, β -factor C was used as an activator, because it was very difficult to completely remove LPS from the enzyme fraction of α -factor C. Unexpectedly, β -factor C did not activate wild-type factor B, even in the presence of LPS (Fig. 2B). Factor B was proteolytically cleaved at two sites within the -Arg¹⁰³-Ser¹⁰⁴- (site 1) and the -Ile¹²⁴-Ile¹²⁵- (site 2) bonds (Fig. 1) (9, 23). Cleavage within the site 1 bond yielded the two-chain form of zymogen, consisting of the L and H chains. On the other hand, cleavage within the site 2 bond to convert the H chain into the B chain was essentially required for the activation of factor B (23). Therefore, activation of factor B is followed by the appearance of the B chain. As expected, α -factor C effectively activated wild-type factor B to induce the appearance of the B chain of factor B in the presence of LPS (Fig. 2C, left panel). In contrast, β -factor C did not cleave the site 2 bond of wild-type factor B (Fig. 2C, right panel) under the same conditions, suggesting that artificial activation of factor C by chymotrypsin causes a loss of its original potency to activate factor B.

Loss of LPS Binding Activity of Wild-type Factor C by Chymotrypsin—The L chain of wild-type factor C was cleaved by incubation with LPS or chymotrypsin and converted into the single band of the B chain, indistinguishable between the two cases by Western blotting (Fig. 3A). To confirm whether the additional proteolytic cleavage(s) of the H chain of factor C occurred during incubation with LPS or chymotrypsin, the time course of the cleavage reaction of the H chain was monitored by Western blotting. Incubation with chymotrypsin, but not with LPS, slightly decreased the apparent molecular weight of the H

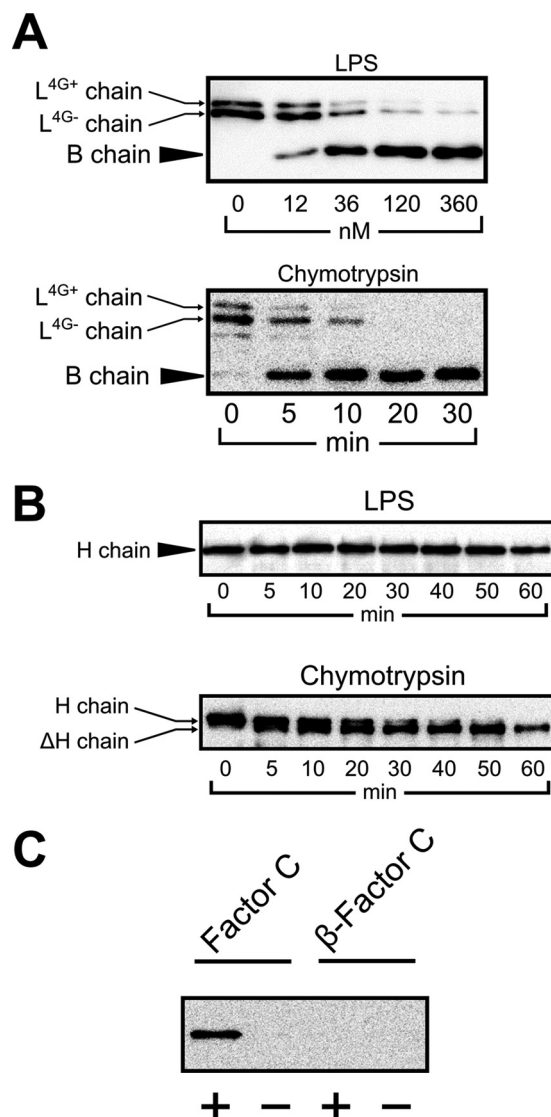


FIGURE 3. Identification of an additional cleavage of wild-type factor C by chymotrypsin. A, wild-type factor C (170 nM) was incubated with several concentrations of LPS for 10 min (top) or 200 nM chymotrypsin for the indicated durations (bottom). Activated factor C was detected by Western blotting with polyclonal antibody. The data are representative of three independent experiments. The fourth N-glycosylation site located in the L chain of factor C was partially modified, and the L^{4G+} and L^{4G-} chains indicate the fourth N-glycosylation site-modified and -unmodified L chains, respectively. B, wild-type factor C (170 nM) was incubated with LPS (0.44 nM) (top) or chymotrypsin (200 nM) (bottom) for several durations, and activated factor C was detected by Western blotting with monoclonal antibody 2C12. The data are representative of three independent experiments. C, binding assays of wild-type factor C and β -factor C to LPS were performed with LPS-immobilized Sepharose (+) or Sepharose as a negative control (-). Monoclonal antibody 2C12 was used in Western blotting. The data are representative of three independent experiments.

chain to that of the Δ H chain (Fig. 3B). The N-terminal sequence analysis of the Δ H chain revealed the sequence of Xaa-Lys-Pro-Xaa-Asp-Asp-Leu- (where Xaa is an unidentified residue by Edman degradation), corresponding to the sequence of Cys⁴¹-Lys⁴²-Pro⁴³-Cys⁴⁴-Asp⁴⁵-Asp⁴⁶-Leu⁴⁷- (Table 1), indicating that additional cleavage by chymotrypsin occurs within the -Tyr⁴⁰-Cys⁴¹- bond, close to the tripeptide motif for LPS recognition. To confirm whether β -factor C retains LPS binding ability, β -factor C was incubated with LPS-immobi-

TABLE 1
The N-terminal sequence analyses of β -factor C

	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7
Amino acid	(Cys)	Lys	Pro	(Cys)	Asp	Asp	Leu
Yield (pmol)	— ^a	6.0	3.0	—	3.2	4.7	4.3

^a —, not detectable.

lized Sepharose. As a result, β -factor C exhibited no significant affinity for LPS-immobilized Sepharose (Fig. 3C). This demonstrated that β -factor C loses the original LPS binding ability of factor C by additional proteolytic cleavage of the -Tyr⁴⁰-Cys⁴¹-bond, despite retaining sufficient amidase activity against the peptide substrate, and that the interaction between α -factor C and LPS is essentially required for the activation of factor B.

An Inhibitory Effect of Triton X-100 on Factor B Activation by α -Factor C in the Presence of LPS—Autocatalytic activation of factor C on LPS is inhibited by adding the synthetic surfactant Triton X-100, especially above the critical micelle concentration of Triton X-100 (0.24 mM) (5, 24). To determine whether Triton X-100 inhibits the activation of factor B by α -factor C, wild-type factor B was incubated with α -factor C in the presence of LPS (0.44 nM) and various concentrations of Triton X-100. Triton X-100 strongly inhibited the activation of wild-type factor B by α -factor C above the critical micelle concentration of Triton X-100 (Fig. 4A). In contrast, Triton X-100 did not inhibit the amidase activity of the activated factor B (Fig. 4B). In addition, monitoring the appearance of the H and B chains of factor B confirmed that, in the presence of Triton X-100, α -factor C cleaved the site 1 bond of wild-type factor B, but not the site 2 bond required for the activation of factor B (Fig. 4C).

Wild-type Factor B Is an LPS-binding Protein—To confirm the LPS binding capacity of factor B, wild-type factor B was incubated with LPS-immobilized Sepharose. Wild-type factor B exhibited LPS binding activity, and free LPS competitively inhibited the binding of wild-type factor B to the LPS-immobilized Sepharose (Fig. 5A). The LPS binding parameters of wild-type factor B were analyzed by surface plasmon resonance analysis (Fig. 5B and Table 2). Moreover, the dissociation constant of wild-type factor B was also determined to be $K_d = 1.03 \times 10^{-8} \pm 0.44 \times 10^{-8}$ M by quartz crystal microbalance analysis (Fig. 5C). These results revealed that wild-type factor B binds to LPS with high affinity comparable with that of factor C.

Identification of the LPS-binding Region of Wild-type Factor B—A mutant factor B without the clip domain (Δ clip-factor B) was prepared, and its LPS binding activity was examined. Δ clip-factor B exhibited no binding activity against LPS-immobilized Sepharose (Fig. 6A). In contrast, the clip domain of factor B (GST-clip) bound to LPS-immobilized Sepharose (Fig. 6B). To clarify the functional role of the clip domain, wild-type factor B or Δ clip-factor B was activated by α -factor C in the presence of LPS, and the resulting amidase activity was measured. Activation of Δ clip-factor B was considerably slower than that of wild-type factor B (Fig. 6C). Consistent with this phenomenon, the B chain appeared much more slowly in the activation of Δ clip-factor B than in that of wild-type factor B (Fig. 6, D and E). These findings indicate that the clip domain of

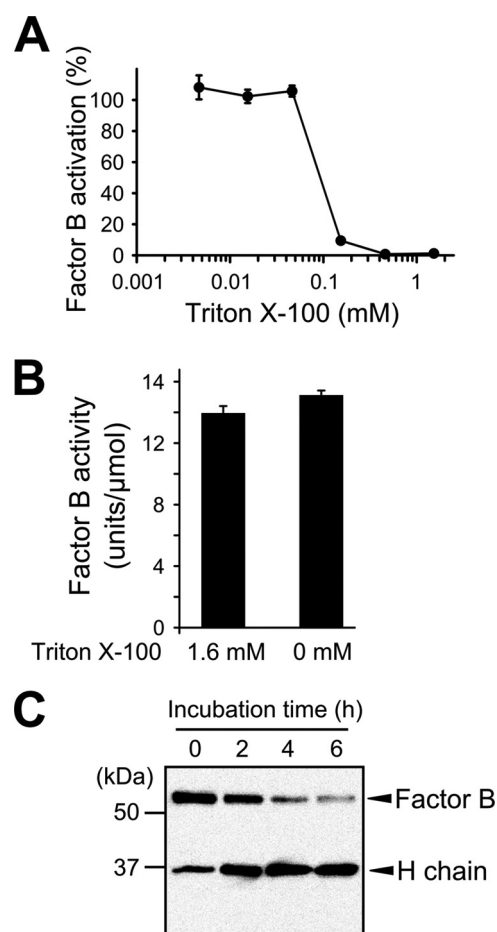


FIGURE 4. The inhibitory effect of Triton X-100 on the activation of factor B by α -factor C. A, wild-type factor B (120 nM) was incubated with α -factor C (0.22 nM) in the presence of LPS (0.44 nM) and several concentrations of Triton X-100 for 5 min, and then factor B activity was measured. B, wild-type factor B (120 nM) was incubated with α -factor C (0.22 nM) in the presence of LPS (0.44 nM). The amidase activity of activated factor B was measured in the presence of Triton X-100 (1.6 mM). The data are the means $\pm$ S.D. of three independent experiments. C, wild-type factor B (120 nM) was incubated with α -factor C (0.22 nM) in the presence of LPS (0.44 nM) and Triton X-100 (1.6 mM) for the indicated durations and then subjected to Western blotting with anti-factor B polyclonal antibody.

factor B contains the LPS-binding region with an essential function in the activation by α -factor C in the presence of LPS.

Discussion

Proteolytic activation of factor B requires cleavage within the -Ile¹²⁴-Ile¹²⁵-bond (site 2) by α -factor C, a comparable reaction to the LPS-induced autocatalytic cleavage within the -Phe⁷³⁷-Ile⁷³⁸-bond of factor C (Fig. 1). We found that wild-type factor B is efficiently activated by α -factor C, but not by β -factor C, through cleavage within the site 2 bond of factor B in an LPS-dependent manner (Fig. 2). We also found that β -factor C loses the original LPS binding ability of factor C during artificial activation by chymotrypsin through cleavage within the -Tyr⁴⁰-Cys⁴¹-bond (Fig. 3). This cleavage site is very close to the -Arg³⁶-Trp³⁷-Arg³⁸-sequence, the unique tripeptide motif for LPS recognition (4), suggesting that this additional cleavage by chymotrypsin disrupts an expected loop structure around the tripeptide motif required for the LPS binding of factor C. The specific activities of α -factor C and β -factor C used in this study

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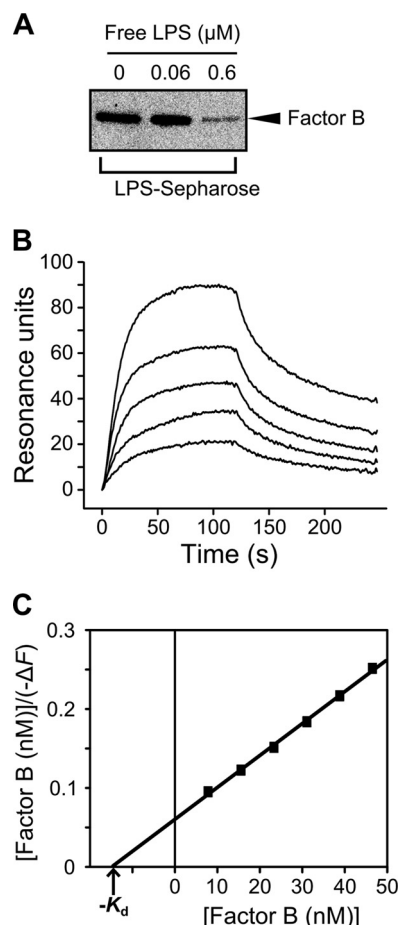


FIGURE 5. Binding activity of wild-type factor B to LPS. *A*, binding activity of wild-type factor B to LPS-immobilized Sepharose was measured in the presence or absence of free LPS. LPS-immobilized Sepharose reacted with wild-type factor B was precipitated by centrifugation and subjected to Western blotting with anti-factor B polyclonal antibody. The data are representative of three independent experiments. *B*, association and dissociation of wild-type factor B with the immobilized biotinylated LPS were determined by surface plasmon resonance analysis. Sensorgrams for the binding of wild-type factor B to the immobilized biotinylated LPS were overlaid at various concentrations of wild-type factor B. *C*, the dissociation constant of wild-type factor B with biotinylated LPS was determined by quartz crystal microbalance analysis. The double-reciprocal plot of $[\text{Factor B}]/(-\Delta F)$ against $[\text{Factor B}]$ gave a straight line with the intercept on the abscissa equal to $-K_d$. The data are representative of three independent experiments.

TABLE 2

LPS binding parameters of wild-type factor C and wild-type factor B by surface plasmon resonance analysis

	k_a $M^{-1}s^{-1}$	k_d s^{-1}	K_d M
Factor C ^a	$4.29 \times 10^6 \pm 2.50 \times 10^6$	$3.25 \times 10^{-3} \pm 0.42 \times 10^{-3}$	7.58×10^{-10}
Factor B	$1.71 \times 10^6 \pm 0.73 \times 10^6$	$5.98 \times 10^{-3} \pm 0.88 \times 10^{-3}$	3.49×10^{-9}

^a These parameters are cited from data obtained in Ref. 5.

were 7.4 and 6.6 units/nmol against Boc-Val-Pro-Arg-pNA in the presence of LPS (0.44 nM), respectively. Interestingly, α -factor C under the same conditions exhibited no amidase activity against a peptide substrate for chymotrypsin, succinyl-Ala-Ala-Pro-Phe-MCA. These findings suggest that a specific conformational state around the site 2 bond of factor B induced by interaction with LPS is crucial for proteolytic activation by α -factor C.

Wild-type factor B exhibited LPS binding activity with the dissociation constant obtained by surface plasmon resonance or quartz crystal microbalance analysis comparable with that of wild-type factor C (Table 2 and Fig. 5), and Δ clip-factor B lost its original LPS binding ability. Moreover, the clip domain of factor B (GST-clip) retained LPS binding activity (Fig. 6B), indicating that factor B is the second LPS-binding protease zymogen in the coagulation cascade after factor C and that the LPS-binding site of factor B is located in the clip domain. In addition, in the presence of LPS, Triton X-100 strongly inhibited activation of wild-type factor B by α -factor C above the critical micelle concentration, suggesting that Triton X-100 mixes into LPS to form an ineffective surface for the proteolytic activation of factor B by α -factor C. To our knowledge, this is the first report of a clip domain with LPS binding affinity. We concluded that the clip domain of factor B has an important function in localizing factor B to the surface of Gram-negative bacteria or LPS released from bacteria to initiate efficient activation by α -factor C.

The mammalian blood coagulation cascade involves several serine-protease zymogens and proceeds to the proteolytic activation, as follows (1): the extrinsic pathway begins with autocatalytic activation of a vitamin K-dependent zymogen factor VII, after which the other vitamin K-dependent zymogens, including factor IX, factor X, and prothrombin, are activated in that order in the presence of protein cofactors of tissue factor, factor V, and factor VIII. These vitamin K-dependent zymogens are all recruited to the reaction center through specific interactions between the N-terminal γ -carboxyglutamic acid-containing domain of each zymogen, coordinating Ca^{2+} ions, and the surfaces of phospholipids released from platelets or tissues at injured sites. This concentration mechanism of protease zymogens on the phospholipid surface has important roles for inducing efficient proteolytic activation at local sites and preventing the coagulation reaction from spreading to the whole body. We found that a similar strategy occurs in horseshoe crab hemolymph coagulation: the cascade begins with autocatalytic activation of an LPS-responsive zymogen factor C, and another LPS-binding zymogen factor B is then activated. The two zymogens are recruited to the reaction center through specific interaction between the N-terminal Cys-rich region of factor C or the N-terminal clip domain of factor B and the surface of LPS released from Gram-negative bacteria. The basic strategies of the two proteolytic cascades triggered in biologic substances to amplify and regulate various biologic reactions must be evolutionarily conserved.

Recently, human caspase-4 and its mouse ortholog caspase-11 (caspase-4/11), which are cysteine-protease zymogens, were identified as intracellular LPS-binding zymogens (25). These are activated in response to the complex formation with LPS internalized into the cytosol to induce pyroptosis and secretion of IL-1 β (26, 27), causing endotoxin shock and sepsis, whereas the molecular activation mechanism of caspase-4/11 on LPS remains unknown. Horseshoe crab hemolymph coagulation is an ideal model system for understanding the reaction mechanism of proteolytic cascades on the surfaces of biologic substances.

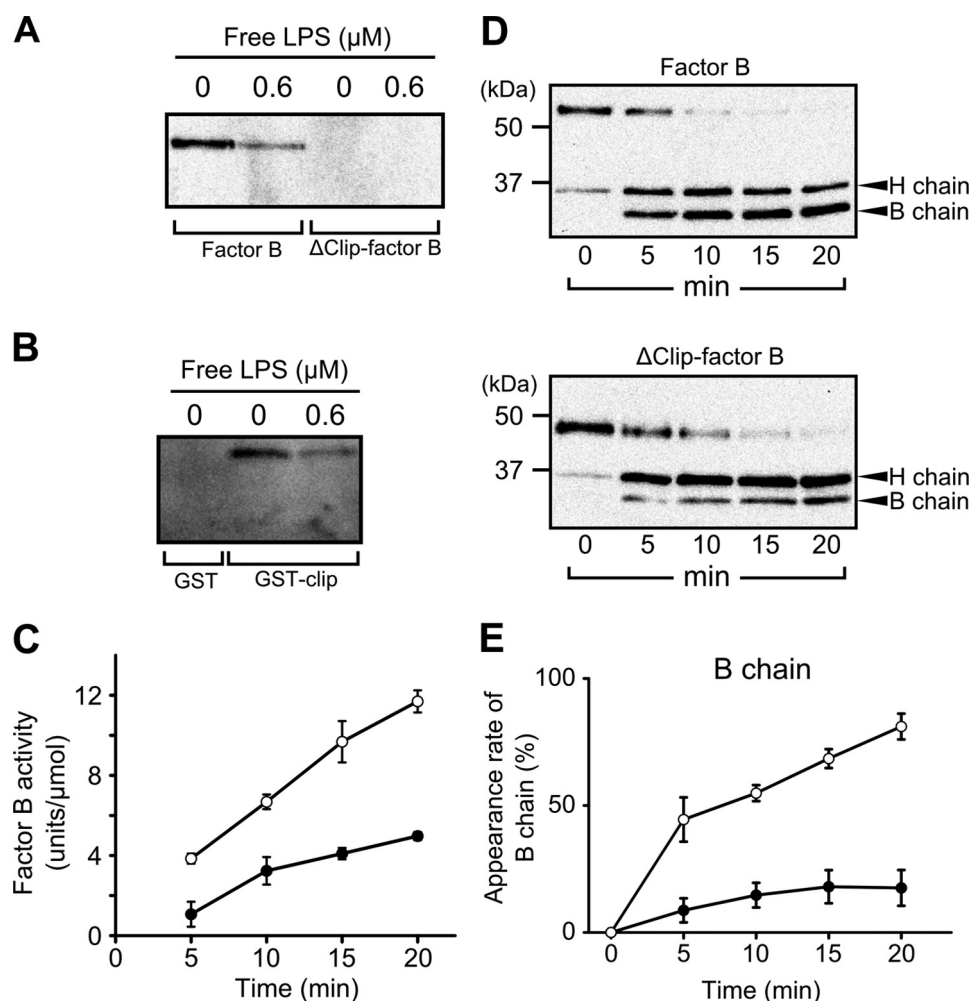


FIGURE 6. LPS-binding assay and activation of Δ clip-factor B. *A*, binding activity of Δ clip-factor B to LPS-immobilized Sepharose was measured in the presence or absence of free LPS. Wild-type factor B was used as a positive control. LPS-immobilized Sepharose reacted with Δ clip-factor B or wild-type factor B was precipitated by centrifugation and subjected to Western blotting with anti-factor B polyclonal antibody. *B*, binding activity of the GST-clip domain to LPS-immobilized Sepharose was measured in the presence or absence of free LPS. GST was used as a negative control. Anti-GST tag HRP-Direct antibody was used in Western blotting. *C*, wild-type factor B or Δ clip-factor B (120 nM) was incubated with α -factor C (0.22 nM) in the presence of LPS (0.44 nM) for the indicated durations, and then the amidase activity generated was assayed. The open and closed circles indicate wild-type factor B and Δ clip-factor B, respectively. *D*, wild-type factor B (top panel) or Δ clip-factor B (bottom panel) was incubated with α -factor C in the presence of LPS (0.44 nM) for the indicated durations. Anti-factor B polyclonal antibody was used in Western blotting. *E*, density of the B chain was analyzed with ImageJ software. The vertical axis shows the appearance rate of the B chain in each lane in *D*. The open and closed circles indicate the B chain of wild-type factor B and Δ clip-factor B, respectively. The data are representative of three independent experiments in *A*, *B*, and *D*. The data are the means $\pm$ S.D. of three independent experiments in *C* and *E*.

A reagent prepared from hemocyte lysates of the horseshoe crab *Limulus polyphemus* was used for a sensitive test to detect endotoxins of Gram-negative bacteria, which is called the “*Limulus* test” (28, 29). We previously reported that wild-type factor C expressed in the HEK293S GnT1[−] cell line has an LPS-induced activity equivalent to that of native factor C (5). Here we reported a functional wild-type factor B expressed in the identical mammalian cell line. We are convinced that applying these recombinants to a next generation *Limulus* test will contribute to the development of more sensitive and convenient methods for detecting endotoxins for a variety of biomedical uses and will help to reduce the use of natural resources.

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