Roles of the clip domains of two proteasezymogens in the coagulation cascade in horseshoe crabs

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The lipopolysaccharide (LPS)-triggered coagulation cascade in horseshoe crabs comprises three proteasezymogens: prochelicerase C (proC), prochelicerase B (proB), and the proclotting enzyme (proCE). The presence of LPS results in autocatalytic activation of proC to α-chelicerase C, which, in turn, activates proB to chelicerase B, converting proCE to the clotting enzyme (CE). ProB and proCE contain an N-terminal clip domain, but the roles of these domains in this coagulation cascade remain unknown. Here, using recombinant proteins and kinetics and binding assays, we found that five basic residues in the clip domain of proB are required to maintain its LPS-binding activity and activation by α-chelicerase C. Moreover, an amino acid substitution at a potential hydrophobic cavity in proB’s clip domain (V55A-proB) reduced both its LPS-binding activity and activation rate. WT proCE exhibited no LPS-binding activity, and the WT chelicerase B-mediated activation of proCE variant with a substitution at a potential hydrophobic cavity (V55A-proCE) was ~4-fold slower than that of WT proCE. The \( K_{cat}/K_m \) value of the interaction of WT chelicerase B with V55A-proCE was 7-fold lower than that of the WT chelicerase B-WT proCE interaction. The enzymatic activities of V55A-chelicerase B and V55A-CE against specific peptide substrates were indistinguishable from those of the corresponding WT proteases. In conclusion, the clip domain of proB recruits it to a reaction center composed of α-chelicerase C and LPS, where α-chelicerase C is ready to activate proB, leading to chelicerase B–mediated activation of proCE via its clip domain.

The horseshoe crab hemolymph contains granular hemocytes, which respond selectively to lipopolysaccharide (LPS) of Gram-negative bacteria by secreting serine proteasezymogens to initiate a proteolytic coagulation cascade (1–4). We previously proposed new terms for proteasezymogens in the horseshoe crab coagulation cascade—prochelicerase C, prochelicerase B, and prochelicerase G, for factor C, factor B, and factor G, respectively—each of which is activated into the corresponding chelicerase (5). The LPS-triggered horseshoe crab coagulation cascade consists of prochelicerase C (proC), prochelicerase B (proB), and the proclotting enzyme (proCE), all of which belong to the trypsin family.

A classical model for the proteolytic activation of serine proteasezymogens has been established for trypsinin and chymotrypsinogen; bothzymogens are activated by proteolytic cleavage of the Arg15–Ile16 bond in chymotrypsinogen numbering, followed by an insertion of the newly appearing N-terminal Ile16 into the activation pocket, the Ile16 cleft, to form a salt bridge between the α-amino group of Ile16 and the side chain of Asp194, which triggers conformational changes of the substrate binding site and the oxyanion hole required for catalysis (6, 7). On the other hand, studies on autocatalytic activation of two thrombin precursors, prethrombin-2 and prothrombin, have been carried out (8, 9). The Arg15–Ile16 bond of prothrombin-2, the immediate precursor of thrombin, is proteolytically cleaved by coagulation factor Xa under physiological conditions, whereas a triple mutant of prethrombin-2 with the substitutions E14A/D14A/E18A has a reduced level of electrostatic interactions between Arg15 and these residues, which promotes autocatalytic activation of the mutant to the mature enzyme spontaneously without the need for the activation by coagulation factor Xa.

The autocatalytic cleavage of proC in the presence of LPS occurs within the Phe237–Ile738 bond, which corresponds to the Arg15–Ile16 bond, to produce an active protease, α-chelicerase C (3, 4, 10). In the absence of LPS, proC is artificially activated by chymotrypsin through cleavage of the Phe237–Ile738 bond with an additional proteolytic cleavage of the Tyr40–Cys41 bond, resulting in its conversion into β-chelicerase C with amidase activity against a specific peptide substrate (11). However, β-chelicerase C possesses neither LPS-binding activity nor proB-activating activity (11).

The autocatalytic cleavage of proC occurs intermolecularly between proC* (an active transition state) molecules in the presence of LPS (5), and the N-terminal Arg4 residue of proC is essential for autocatalytic activation (12). The active transition state proC* does not exhibit intrinsic chymotryptic activity against the Phe237–Ile738 bond, but it may recognize a threedimensional structure around the cleavage site (5). The resulting α-chelicerase C activates proB to chelicerase B through proteolytic cleavage of the Ile124–Ile125 bond (13), and then chelicerase B converts proCE to the clotting enzyme (CE) through cleavage of the Arg98–Ile99 bond (14) (Fig. 1). An LPS-binding site of proC is located in a tripeptide sequence (Arg36–Trp37–Arg39) in the N-terminal cysteine-rich domain with a dissociation constant \( (K_d) \) of 7.6 × 10⁻¹⁰ M (15).

Instead of the N-terminal cysteine-rich domain found in proC, proB and proCE contain an N-terminal clip domain (13, 14). The clip domain was first identified in proCE (14); later, clip domain-containing proteases or protease homologs were identified in arthropods, such as those involved in dorsal-ventral polarity in Drosophila and the proteolytic cascades of

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Roles of the clip domains in protease zymogens

Prochelicerase B

![Schematic domain structures of proB and proCE.](image)

Figure 1. Schematic domain structures of proB and proCE. The two interchain disulfide bonds of proB and proCE are indicated by bars, and diamonds show N-linked glycosylation sites of proB and proCE (13, 14).

Proclotting enzyme

The single-chain form of proB is cleaved at site 1, and the cleaved B chain is further cleaved at site 2 by an unknown protease (Fig. 1A). The activation rate of both types of proB was assessed by detecting the appearance of the B chain through cleavage at site 2 by Western blotting using an anti-B-chain antibody (11).

To confirm whether the cleavage reaction at site 2 for the proteolytic activation was quantified by Western blotting, WT proB at various concentrations was incubated with α-chelicerase C in the presence of LPS at an [E]/[S] ratio of 1/200 for 30 min at 37°C and subjected to Western blotting. Site 2 of WT proB was cleaved, and the resulting B chain was detected (Fig. 2A). The relative band density was in direct proportion to the concentration of WT proB at least in the density range between 25 and 100 nM (Fig. 2B). After 30 min of incubation, WT proB at every concentration was cleaved ~35% under the conditions used (Fig. 2C).

Basic residues in the clip domain of proB are required to maintain the rate of activation by α-chelicerase C

Studies on LPS-binding proteins such as proC (14), horseshoe crab anti-LPS factor (25), human MD2 (26, 27), and mammalian caspases 4, 5, and 11 (28) demonstrated that specific basic residues in their amino acid sequences are required for the interaction with LPS. The clip domain of proB contains five basic residues (Arg17, Lys21, Lys25, Lys38, and Arg50). To determine whether the basic residues in the clip domain of proB are involved in the interaction with LPS, substitution mutants were prepared by replacing each of these basic residues with a glutamic acid, R17E-proB, K21E-proB, K25E-proB, K38E-proB, and R50E-proB.

Each of these substitution mutants was incubated with WT α-chelicerase C in the presence of LPS and subjected to Western blotting (Fig. 3A). All of the mutants, especially K21E-proB, K25E-proB, and K38E-proB, exhibited slower activation by α-chelicerase C at the initial reaction phase, around 2 min of incubation, than WT proB (Fig. 3, A and B). To confirm the reduction of the activation rates of K21E-proB, K25E-proB, and K38E-proB by Western blotting, the expression time course of the amidase activity was monitored using a fluorogenic peptide substrate for chelicerase B, butoxycarbonyl-Leu-Thr-Arg-4-methylcoumaryl-7-amide (Boc-L-T-R-MCA). The amidase activities of the three mutants were expressed more slowly than that of WT proB (Fig. 3C). These findings indicate that the basic residues in the clip domain of proB play important roles to maintain the activation rate by α-chelicerase C.

The basic residues in the clip domain of proB are required to maintain the LPS-binding activity of proB

To determine the LPS-binding activity of these mutants of proB, the LPS-binding parameters were determined by surface plasmon resonance (SPR) analysis. As a positive control, WT proB exhibited an LPS-binding activity with a dissociation constant (Kd) of 1.2 × 10^-9 M (kdiss = 6.0 × 10^5 M^-1 s^-1 and kdiss = 7.6 × 10^-3 s^-1), comparable to the reported Kd value of 3.5 × 10^-9 M (11). However, the binding parameters of the five point

**Results**

Quantification of proteolytic activation of proB by Western blotting

WT proB, prepared in an HEK293S cell line lacking N-acetylglucosaminyltransferase I (GnTI'), contains two types of zymogen: the single-chain form and the two-chain form, consisting of the H and L chains formed through cleavage at site 1 by an unknown protease (Fig. 1). The activation rate of both types of proB by α-chelicerase C is assessed by detecting the appearance of the B chain through cleavage at site 2 by Western blotting using an anti-B-chain antibody (11).

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mutants of proB could not be determined by SPR analysis because of their lower affinity for the LPS used to coat the sensor tip. These findings indicate that the basic residues in the clip domain of proB play an important role in maintaining the LPS-binding activity.

Determination of kinetic parameters for the activated forms of K21E-proB, K25E-proB, and K38E-proB against the peptide substrate Boc-L-T-R-MCA

K21E-proB, K25E-proB, and K38E-proB were fully converted into the active forms, which required a 6-h incubation at 37 °C with WT α-chelicerase C in the presence of LPS, and the kinetic parameters were determined using the specific fluorogenic substrate Boc-L-T-R-MCA. A preliminary experiment using WT chelicerase B showed a very high $K_m$ value ($\sim 200 \mu M$). Therefore, only $k_{cat}/K_m$ values were determined under the conditions where [S] was below the $K_m$ value at an enzyme concentration of 10 nM, as described under “Experimental Procedures.”

The $k_{cat}/K_m$ values of these activated mutants were equivalent to that of WT chelicerase B (Table 1), indicating that the substitutions of Lys21, Lys25, and Lys38 with E did not affect the amidase activities against the peptide substrate.

A potential hydrophobic cavity in the clip domain of proB is required to maintain the activation rate by α-chelicerase C and the LPS-binding activity of proB

Crystal structural analyses of the clip domains in insects were reported for prophenoloxidase-activating factor II of the beetle Holotrichia diomphalia (Hd-PPAF-II) (29), grass protease zymogen of the fly Drosophila melanogaster (30), and prophenoloxidase-activating proteinase-2 of the moth Manduca sexta (31). These analyses revealed a unique hydrophobic cavity in the clip domains, comprising several conserved hydrophobic amino acids (dots in Fig. 4A).

The hydrophobic cavity of Hd-PPAF-II is very important for recognizing prophenoloxidase, because an Hd-PPAF-II mutant with the substitution V111A causes a deficiency of the cofactor activity needed for the proteolytic activation of prophenoloxidase (29). Therefore, we prepared a substitution mutant in the clip domain of proB (V55A-proB), which corresponds to residue Val111 of Hd-PPAF-II (Fig. 4A). Based on the sequence similarity, the locations of Val55 and the five basic residues in the clip domain of proB were superimposed in the crystal structure of the clip domain of Hd-PPAF-II (Fig. 4B).

The activation of V55A-proB by WT α-chelicerase C in the presence of LPS was monitored by both Western blotting and the expression time course of the amidase activity. Western blotting indicated that the relative band intensity of the B chain for V55A-proB at 5 min of incubation was 4-fold lower than that for WT proB (Fig. 5, A and B). The expression of the amidase activity for V55A-proB at 5 min of incubation was 6-fold lower than that for WT proB (Fig. 5C).

V55A-proB was fully converted to V55A-chelicerase B by 6 h of incubation with WT α-chelicerase C in the presence of LPS. The $k_{cat}/K_m$ value of V55A-chelicerase B against Boc-L-T-R-MCA was similar to that of WT chelicerase B.
MCA was equivalent to that of WT chelicerase B, suggesting that the potential hydrophobic cavity of proB does not affect the amidase activity (Table 1).

Interestingly, V55A-proB exhibited no binding activity for the LPS-coated tip of the SPR sensor, indicating that the potential hydrophobic cavity plays a role in maintaining the LPS-binding activity of proB.

Preparation of WT proCE and the LPS-binding assay of WT proCE by SPR analysis

ProCE is another clip domain-containing zymogen in the coagulation cascade. Native proCE purified from horseshoe crab hemocytes is a single-chain glycoprotein with an apparent molecular mass of 54 kDa on SDS-PAGE (14, 32). In the present study, WT proCE was prepared in HEK293S GnTI cells, which produces recombinants with a restricted and homogeneous N-glycan of Man5GlcNAc2 (33). The prepared WT proCE had a single band with an apparent molecular mass of 48 kDa on SDS-PAGE (Fig. 6A).

WT proCE had no significant affinity for the LPS-coated tip of the SPR sensor, indicating that the clip domain of proCE has no LPS-binding activity. The LPS-dependent autocatalytic activation of proC is inhibited by Triton X-100 above the critical micelle concentration at 0.24 mM (12). To examine the effect of Triton X-100 on the activation of proCE by chelicerase B in the presence of LPS, Triton X-100 was added to the reaction mixture of WT proCE (50 nM) and WT chelicerase B (1.3 nM) in the presence of LPS (10 nM). Triton X-100 at 0.8 mM did not inhibit

Table 1

| Protease          | Substrate     | $K_{m}$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_{m}$ (M$^{-1}$ s$^{-1}$) |
|-------------------|---------------|--------------|----------------------|------------------------------------|
| WT $\alpha$-chelicerase C | Boc-V-P-R-MCA  | 93 ± 10      | 34 ± 1               | 370,000 ± 33,000                    |
| WT chelicerase B  | Boc-L-T-R-MCA  | ND           | 5,300 ± 7            |                                    |
| K21E-chelicerase B | Boc-L-T-R-MCA  | ND           | 5,000 ± 10           |                                    |
| K25E-chelicerase B | Boc-L-T-R-MCA  | ND           | 4,900 ± 40           |                                    |
| K38E-chelicerase B | Boc-L-T-R-MCA  | ND           | 5,100 ± 47           |                                    |
| V55A-chelicerase B | Boc-L-T-R-MCA  | ND           | 5,100 ± 47           |                                    |
| WT CE             | Boc-L-G-R-MCA  | 55 ± 6       | 194 ± 11             | 3,600,000 ± 410,000                |
| Δclip-CE          | Boc-L-G-R-MCA  | 47 ± 7       | 200 ± 9              | 4,300,000 ± 280,000                |
| V53A-CE           | Boc-L-G-R-MCA  | 46 ± 7       | 182 ± 3              | 4,000,000 ± 610,000                |

Data are means ± S.E. from three independent experiments. ND, not determined.

Figure 3. The basic residues in the clip domain of proB are required to maintain the rate of activation by $\alpha$-chelicerase. A, WT proB, R17E-proB, K21E-proB, K25E-proB, K38E-proB, or R50E-proB (100 nM) was incubated with WT $\alpha$-chelicerase C (1.5 nM) in the presence of LPS (60 nM) at 37 °C, and at the indicated times, aliquots were subjected to Western blotting using anti-B chain antibody. Data are representative of three independent experiments. B, the density of the S, H, and B chains was analyzed with Image J software. The vertical axis shows the appearance rate of the B chain in each mutant. WT proB (open circles), R17E-proB (closed circles), K21E-proB (open triangles), K25E-proB (closed triangles), K38E-proB (open squares), R50E-proB (closed squares), C, WT-proB (open circles), K21E-proB (open triangles), K25E-proB (closed triangles) or K38E-proB (open squares) (150 nM) was incubated with WT $\alpha$-chelicerase C (1.0 nM) in the presence of LPS (40 nM), and amidase activities of the resulting WT chelicerase B and the activated mutants were assayed at the indicated times using Boc-L-T-R-MCA. Units of amidase activity were defined as micromoles of the digested synthetic substrate per minute, and specific activity was expressed as units per micromole of WT proB or the mutants. Data in panels B and C are the means ± S.D. of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

Roles of the clip domains in protease zymogens
the activation of WT proCE under the conditions used, suggesting that LPS is not a requirement for the proteolytic activation of proCE by chelicerase B.

**The potential hydrophobic cavity in the clip domain of proCE is required to maintain the rate of activation by chelicerase B**

WT proCE was incubated at 37 °C with WT chelicerase B at an [E]/[S] ratio of 1/17, and the time course of the proteolytic conversion of proCE to CE was evaluated by the appearance of the H chain on Western blotting using anti-H-chain antibody. WT proCE was almost fully converted to WT CE by 15 min of incubation under the conditions used (Fig. 6B, left panel). To examine whether the clip domain of proCE plays an important role in the activation by chelicerase B, we prepared a deletion mutant of the clip domain, Dclip-proCE. The relative band intensity of H chain for Dclip-proCE at 5 min of incubation was 3-fold lower than that for WT proCE (Fig. 6B, middle panel, and Fig. 6C). To confirm the reduction of the activation rate of Dclip-proCE by WT chelicerase B, the expression time course of the amidase activity was monitored using a specific peptide substrate, Boc-L-G-R-MCA, indicating that the activation rate of Dclip-proCE was ~7-fold lower than that of WT proCE (Fig. 6D).

We prepared proCE mutant with the substitution V53A in the clip domain (V53A-proCE), which corresponds to Val135 of proB, the component of the potential hydrophobic cavity. The time course of the conversion of V53A-proCE to V53A-CE was also evaluated by both Western blotting (Fig. 6B, right panel, and Fig. 6C).
Roles of the clip domains in protease zymogens

A

(kDa)

0 1 2 5 10 15

B

(min)

0 1 2 5 10 15

WT proCE

Δclip-proCE

V53A-proCE

Figure 6. Comparison of the activation rate of Δclip-proCE or V53A-proCE with that of WT proCE. A, purified WT proCE was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. An arrowhead indicates WT proCE on SDS-PAGE, B, WT proCE, Δclip-proCE, or V53A-proCE (50 nM) was incubated with WT chelicerase B (3.0 nM) and LPS (6.0 nM), and amidase activities of WT proCE, Δclip-CE, and V53A-CE were assayed at the indicated times using Boc-L-G-R-MCA. Units of amidase activity were defined as nanomoles of the synthetic substrate digested per minute, and specific activity was expressed as units per nanomole of WT proCE (open circles), Δclip-proCE (closed circles) or V53A-proCE (open triangles). Data in panels C and D are means ± S.D. from three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001.

and Fig. 6C) and the amidase activity against the specific peptide substrate (Fig. 6D). Based on the expression of amidase activity for the resulting V53A-CE, the proteolytic conversion rate observed for V53A-proCE was about 4-fold lower than that observed for WT proCE (Fig. 6D), indicating that the potential hydrophobic cavity of proCE is required to maintain the rate of activation by chelicerase B.

**Determination of kinetic parameters for WT CE, Δclip-CE, and V53A-CE against the peptide substrate Boc-L-G-R-MCA**

WT proCE was fully converted to WT CE by 1 h of incubation with WT chelicerase B. On the other hand, Δclip-proCE and V53A-proCE were fully converted to the activated forms by 2 h of incubation with WT chelicerase B. The kinetic parameters were determined using Boc-L-G-R-MCA. A preliminary experiment showed that the $K_m$ value of WT CE was about 50 μM, and therefore, substrate concentrations between 6.25 μM and 100 μM were used at an enzyme concentration of 50 μM. The $K_m$ and $k_{cat}$ values of Δclip-CE and V53A-CE were comparable to those of WT CE (Table 1). These results indicate that the clip domain of CE has no effect on the amidase activity against the peptide substrate.

It is noteworthy that the amidase activity of WT CE against the fluorogenic substrate was higher than those of the other pro tease activities in the horseshoe crab coagulation cascade; the $k_{cat}/K_m$ value of WT CE was 10-fold and 700-fold higher than those of WT α-chelicerase C (12) and WT chelicerase B (Table 1) against their specific synthetic substrates, respectively.

**Determination of kinetic parameters for WT chelicerase B against two protein substrates, WT proCE and V53A-proCE**

The kinetic parameters for WT chelicerase B were determined against WT proCE and V53A-proCE, as described under “Experimental Procedures.” The $K_m$ value of WT chelicerase B against V53A-proCE was 1.8-fold higher than that against WT proCE, and the $k_{cat}$ value of WT chelicerase B against V53A-proCE was 4-fold lower than that against WT proCE (Table 2). Consequently, a 7-fold decrease in the $k_{cat}/K_m$ value against V53A-proCE was calculated, compared with that against WT proCE. These results indicate that the clip domain of proCE is important to enhance the ES complex formation and to increase the catalytic turnover number of chelicerase B.

**Discussion**

The LPS-binding site in the N-terminal cysteine-rich region of proC contains the tripeptide sequence with an aromatic residue immediately flanked by two basic residues (Arg–Trp–Arg) (15), and this motif appears in other LPS-binding proteins, such as human bactericidal permeability-increasing protein (34) and Limulus anti-LPS factor (25, 35). The substitution mutants R36E/R38E-proC and W37A-proC result in the loss of LPS-binding activity of proC (15). Therefore, in the LPS-binding model of proC, the basic side chains of Arg and Arg interact with the phosphates in the lipid A portion of LPS, and the hydrophobic side chain of Trp stacks a hydrophobic surface of the pyranose ring of the glucosamine in the lipid A molecule through the π–π interaction (15).

The substitution mutations at the five basic residues and the Val residue in the clip domain of proB caused the loss of the LPS-binding activity, as assessed by SPR analysis. The basic residues, such as Lys, Lys, and Arg, were estimated to be three-dimensionally located on either side of the hydrophobic Trp residue (Fig. 4B). Therefore, the clip domain of proB may have a similar structural strategy to bind to the lipid A molecule of LPS. However, in this study, each of the five basic residues of proB—i.e., Arg, Lys, Lys, and Arg—was replaced with a Glu residue, resulting in the opposite charge at the substitution site, which may have had different effects on the functional activities, compared with the Ala replacement.

Based on SPR analysis, WT proCE exhibited no LPS-binding activity. Interestingly, three of the five basic residues required for LPS binding in proB, Lys, and Arg, are replaced with Glu, Asn, and Gly, respectively, in proCE (Fig. 4A). These substitutions possibly reflect a characteristic of the clip domain of proCE with no LPS-binding activity. V53A-proB resulted in a reduction of the activation rate by α-chelicerase C (Fig. 5, A and B), and V53A-proCE also exhibited a reduced rate of activation by chelicerase B, compared with that of the WT proCE (Fig. 6). Moreover, the $k_{cat}/K_m$ value of WT chelicerase B against V53A-proCE was 7-fold lower than that against WT proCE (Table 2). These findings suggest that the potential hydrophobic cavity of the clip domain of proB or proCE functions in proteolytic activation by the corresponding
upstream protease in the coagulation cascade, possibly by enhancing the formation of a favorable ES complex between $\alpha$-chelicerase C and proB or between chelicerase B and proCE and also by increasing the catalytic turnover numbers of $\alpha$-chelicerase C and chelicerase B. The clip domain of the protease zymogen Hd-PPAF-I is also reported to have an essential role in its rapid activation (29).

The kinetic parameters of the amidase activities of the activated mutants, including K21E-chelicerase B, K25E-chelicerase B, K38E-chelicerase B, V55A-chelicerase B, V53A-CE, and Dclip-CE, were equivalent to those of the corresponding WT enzymes (Table 1), indicating that the clip domains of chelicerase B and CE do not affect their catalytic efficiency against the peptide substrates.

Fig. 7 shows a proposed model for the roles of the clip domains of proB and proCE in the coagulation cascade. ProC forms a homooligomer in the presence of LPS (15), and the N-terminal R and the distance between the N terminus and the tripeptide motif are essential elements for oligomer formation (12). The resulting oligomer of proC is converted into the active transition state, proC*, leading to autocatalytic conversion to $\alpha$-chelicerase C (5). ProB is recruited to a reaction center composed of $\alpha$-chelicerase C and LPS through the clip domain containing the basic residues and the potential hydrophobic cavity to accelerate the formation of an ES complex between $\alpha$-chelicerase C and proB to produce chelicerase B. Then, the potential hydrophobic cavity in the clip domain of proCE accelerates the formation of another ES complex between chelicerase B and proCE to produce CE, thereby promoting hemolymph coagulation. Therefore, the clip domains of proB and proCE play essential roles in the zymogen-LPS or the protease-substrate interaction to control the activation of the coagulation cascade in response to infection by Gram-negative bacteria at local sites of injury.

WT proC (12) and proB (11) expressed in HEK293S GnTI cells exhibit procoagulant activities equivalent to those of native proC and proB. In the present study, we prepared WT proCE expressed in the same cell line. WT proCE was fully converted into the active form by WT chelicerase B (Fig. 6). The kinetic parameters of the resulting WT CE against the specific peptide substrate demonstrated an extremely high amidase activity compared with those of WT $\alpha$-chelicerase C and WT chelicerase B (Table 1). We conclude that WT proCE with high procoagulant activity was successfully prepared.

Therefore, the three recombinant protease zymogens in the LPS-triggered coagulation cascade, proC, proB, and proCE, could be applied to a next-generation Limulus test to contribute to the development of sensitive and convenient assays for the detection of LPS for a variety of biomedical uses. These recombinant coagulation factors will also help to reduce the use of native ones prepared from hemocytes of the endangered horseshoe crabs.

### Table 2

| Substrate | $K_{\text{m}}$ (nM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_{\text{m}}$ (M$^{-1}$ s$^{-1}$) |
|-----------|---------------------|--------------------------|-----------------------------------------------|
| WT proCE  | 190 ± 45            | 0.04 ± 0.005             | 200,000 ± 30,000                              |
| V53A-proCE| 340 ± 45            | 0.01 ± 0.001             | 30,000 ± 800                                  |

Data are means ± S.E. from three independent experiments.

### Experimental procedures

#### Materials

HEK293S GnTI$^-$ cells were obtained from the ATCC. LPS derived from *Salmonella enterica* serovar Minnesota R595 (Re) was purchased from List Biological Laboratories, Inc. (Campbell, CA) and used for proB and proCE activation assays. An average molecular weight of 2,500 for R595 (Re) LPS was used for determination of the molar concentration. Biotinylated LPS derived from *Escherichia coli* O111:B4 was purchased from...
Roles of the clip domains in protease zymogens

InvivoGen (San Diego, CA) and used in the SPR analysis for the determination of binding parameters. Peptide substrates were purchased from Peptide Institute, Inc. (Osaka, Japan).

Cloning and mutagenesis of proB and proCE

Full-length DNA fragments of proB and proCE derived from the horseshoe crab *Tachypleus tridentatus* were subcloned into the vector pCA7 (13, 36, 37), and a sequence derived from pHLecc was used as a secretion signal sequence (38). A His tag was added to the C-terminal end of proB by site-directed mutagenesis using inverse PCR. To prepare Δclip-proCE, the full-length proCE contained the cleavage site for PreScission protease between the clip domain and linker region (the ProS–Lys bond).

Expression and purification of recombinant proteins

Recombinant proteins of proC, proB, proCE, proB mutants, and proCE mutants were expressed and purified as described previously (11). In brief, culture media containing recombinant proteins 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 × 5.0 cm). The column was washed with 50 mM NaH₂PO₄-NaOH, pH 8.0, containing 150 mM NaCl and 10 mM imidazole, and the recombinant proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 50–200 mM imidazole. The N-terminal region containing the His tag and the clip domain of proCE was cut off by PreScission protease. The resulting samples were exchanged with 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl at 37 °C in the presence of LPS (8.0 mM), and applied to a nickel-nitrilotriacetic acid-agarose column containing 150 mM NaCl. WT proB and proB mutants (50 nM) were activated by WT chelicerase B (3.0 nM) was incubated with WT proCE or proCE mutants (50 nM). The amidase activities of the aliquots at the indicated times were assayed with 0.4 mM Boc-L-G-R-MCA as described under “Activation of WT proB and proB mutants.”

Western blotting

Samples were subjected to SDS-PAGE in 12% slab gel and transferred to a PVDF membrane. After blocking with 5% skim milk, the membrane was incubated at room temperature for 1 h with polyclonal antibody against the B chain of chelicerase B (11) or the H chain of CE (40) and then with the secondary antibody (HRP-conjugated goat anti-rabbit IgG; Bio-Rad), followed by development with Western Bright Sirius or ECL (Advansta, Menlo Park, CA).

Kinetic analysis of WT α-chelicerase, WT chelicerase B, WT CE, and the mutants of chelicerase B and CE against peptide substrates

Proteases, including WT α-chelicerase C, WT chelicerase B, WT CE, and the mutants of chelicerase B and CE, were incubated with their specific MCA substrates at 37 °C in 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.1 mg/ml of BSA. The initial rate of hydrolysis was measured fluorometrically with excitation at 380 nm and emission at 440 nm after addition of the substrate solution. A kinetic parameter ($k_{cat}/K_m$) for WT chelicerase B or the mutants of chelicerase B was determined for [S] values below the $K_m$ value ($[S] = 12.5\mu M, 25\mu M$, and $50\mu M$) at an enzyme concentration of 10 nM. When these conditions were used, the Michaelis-Menten equation $v = k_{cat}[E][S]/(K_m + [S])$ reduces to $k_{cat}/K_m = v/[E][S]$, since the sum total of $K_m + [S]$ was almost equal to $K_m$ (41). On the other hand, kinetic parameters for WT α-chelicerase C, WT CE, or the mutants of CE were determined by direct linear plotting (42) using the substrate concentrations of $12.5\mu M$, $25\mu M$, $50\mu M$, $100\mu M$, and $150\mu M$ for WT α-chelicerase C at an enzyme concentration of 2 nM and concentrations of $6.25\mu M$, $12.5\mu M$, $25\mu M$, $50\mu M$, and $100\mu M$ for CE or the mutants of CE at an enzyme concentration of 50 pm.

Kinetic analysis of WT chelicerase B against two protein substrates, WT ProCE and V53A-proCE

WT chelicerase B was incubated with WT proCE or V53-proCE at 37 °C in the cuvette of a spectrofluorometer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mg/ml of BSA, and $200\mu M$ Boc-L-G-R-MCA, the specific peptide substrate for CE. The hydrolysis of the peptide substrate by the resulting WT CE or V53A-CE was measured continuously, and the rates of the hydrolysis of the peptide substrate were calculated by tangential velocities of the hydrolysis curves. The concentrations of WT CE and V53A-CE converted from the corresponding zymogens by WT chelicerase B were calculated using the $k_{cat}$ value of WT CE or V53A-CE as follows. Under the conditions where $[S] = Boc-L-G-R-MCA$ is greater than $K_m$ (the $K_m$ value of $E = WT CE$ or V53A-CE against the peptide substrate was ~50 μM, as shown in Table 1), $v = k_{cat}[E][S]/(K_m + [S])$ reduces to $v = k_{cat}[E]_0$, since the sum total of $K_m + [S]$ was almost equal to $[S]$ (41). $[E]_0$, equal to $[WT CE$ or V53A-CE], was calculated using their $k_{cat}$ values. Kinetic parameters for WT chelicerase B against WT proCE and V53A-proCE were...
determined by direct linear plotting (42) using the protein substrate concentrations 100 nM, 150 nM, 200 nM, 300 nM, and 400 nM at an enzyme concentration of 1.0 nM.

**Statistical analysis**

Statistical analysis was performed by unpaired t tests. p values of <0.05 were considered statistically significant.

**LPS-binding assay with SPR analysis**

Biotinylated LPS (0.1 μg) in 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl was immobilized on a sensor chip SA of a Biorec X system (GE Healthcare), according to the manufacturer’s specifications. Recombinant proteins were injected at a flow rate of 30 μl/min in the running buffer, 20 mM Tris-HCl (pH 8.0), containing 150 mM NaCl. The change in the mass concentration on the sensor chip was monitored as a resonance signal by using the program supplied by the manufacturer. Sensorsgrams of the interactions obtained by using the various concentrations of recombinant proteins (approximately 10–100 nM) were analyzed by the BLAevaluation program, version 3.0.

**Data availability**

All data are contained within the manuscript.

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**Abbreviations**—The abbreviations used are: LPS, lipopolysaccharide; Boc-L-T-R-MCA, butoxycarbonyl-Leu-Thr-Arg-4-mehtylecounaryl-7-amide; CE, clotting enzyme; GnTT, lacking N-acetylglucosaminyltransferase I; Hsl-PAF-II, Holotrichia diomphalia prophenoloxidase-activating factor II; proB, prochelicerate B; proCE, proclotting enzyme; SPR, surface plasmon resonance.

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