Staphylococcal Superantigen-like Protein 10 (SSL10) Inhibits Blood Coagulation by Binding to Prothrombin and Factor Xa via Their γ-Carboxyglutamic Acid (Gla) Domain*

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Saotomo Itoh†§, Ryosuke Yokoyama†, Go Kamoshida†, Toshinobu Fujiwara†, Hiromi Okada‡, Takemasa Takii†, Tsutomu Tsuji†, Satoshi Fuji‡, Hideki Hashizume§, and Kikuo Onozaki‡§

From the †Department of Molecular Pathobiology and Therapeutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1, Tanabe-Dori, Mizuho-ku, Nagoya 467-8603, Japan, the ‡Department of Microbiology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan, the §Department of Molecular and Cellular Pathobiology and Therapeutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1, Tanabe-Dori, Mizuho-ku, Nagoya 467-8603, Japan, and the ¶Laboratory of Disease Biology, Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

Background: Staphylococcal superantigen-like proteins (SSLs) share structural similarity with superantigens but no superantigenic activity. Their functions remained unclear.

Results: SSL10 binds to prothrombin and factor Xa via the γ-carboxyglutamic acid domain. SSL10 inhibits the penultimate step of plasma clotting. SSL10 slightly inhibits clotting by coagulase.

Conclusion: SSL10 inhibits blood coagulation.

Significance: This work presents a novel function of SSLs, disturbing blood coagulation.

The staphylococcal superantigen-like protein (SSL) family is composed of 14 exoproteins sharing structural similarity with superantigens but no superantigenic activity. Target proteins of four SSLs have been identified to be involved in host immune responses. However, the counterparts of other SSLs have been functionally uncharacterized. In this study, we have identified porcine plasma prothrombin as SSL10-binding protein by affinity purification using SSL10-conjugated Sepharose. The resin recovered the prodomain of prothrombin (fragment 1 + 2) as well as factor Xa in pull-down analysis. The equilibrium dissociation constant between SSL10 and prothrombin was 1.36 × 10⁻⁷ M in surface plasmon resonance analysis. On the other hand, the resin failed to recover γ-carboxyglutamic acid (Gla) domain-less coagulation factors and prothrombin from warfarin-treated mice, suggesting that the Gla domain of the coagulation factors is essential for the interaction. SSL10 prolonged plasma clotting induced by the addition of Ca²⁺ and factor Xa. SSL10 did not affect the protease activity of thrombin but inhibited the generation of thrombin activity in recalcified plasma. S. aureus produces coagulase that non-enzymatically activates prothrombin. SSL10 attenuated clotting induced by coagulase, but the inhibitory effect was weaker than that on physiological clotting, and SSL10 did not inhibit protease activity of staphylothrombin, the complex of prothrombin with coagulase. These results indicate that SSL10 inhibits blood coagulation by interfering with activation of coagulation cascade via binding to the Gla domain of coagulation factor but not by directly inhibiting thrombin activity. This is the first finding that the bacterial protein inhibits blood coagulation via targeting the Gla domain of coagulation factors.

Staphylococcal superantigen-like proteins (SSLs) are a family of exoproteins composed of 14 members that have molecular masses of 25–35 kDa produced by a commensal human pathogen, Staphylococcus aureus. SSL1–SSL11 are encoded in the staphylococcal pathogenicity island 2 (SaPI2), and SSL12–SSL14 are in the immune evasion cluster (1). The distinctive features of SSLs are structural similarities with staphylococcal superantigens (TSST-1 (toxic shock syndrome toxin 1) and enterotoxins); i.e. all of them are composed of an N-terminal oligonucleotide/oligosaccharide-binding fold and the C-terminal β-grasp structure (2) despite their sequence similarity of less than 40%, and SSLs do not exhibit superantigenic activity (3–5). Recent studies have revealed that several SSLs interact with host immune factors and disturb their functions: SSL7 binds to IgA and inhibits IgA-FcγR interaction (6); SSL7 binds to complement C5 and inhibits the cleavage of C5 and generation of anaphylatoxin C5a (7); SSL5 binds to PSGL-1 (P-selectin glycoprotein ligand-1), which is expressed on leukocytes and involved in rolling of leukocyte on vascular endothelium (8); SSL5 binds to chemokine and anaphylatoxin receptors and antagonizes these chemoattractants (9); SSL5 binds to platelet receptors GPIIb and GPIIIa and inhibits aggregation of platelets (10); SSL5 binds to matrix metalloproteinase-9 and inhibits its protease activity and transmigration of neutrophils through Matrigel basement membrane (11); SSL10 binds to...
CXCR4 and inhibits migration of T cell leukemia (12); SSL10 binds to human IgG, interferes with the interaction between IgG and complement C1q, and inhibits the classical complement activation pathway (13) and Fc receptor-mediated phagocytosis of neutrophils (14); and SSL3 binds to TLR2 (Toll-like receptor 2) and inhibits cytokine production from macrophages in response to TLR2 ligands (15, 16).

We have identified target proteins of SSL3, SSL5, and SSL10 by affinity purification using the Sepharose covalently conjugated with recombinant SSLs from porcine spleen, human neutrophils, and human plasma, respectively (11, 13, 15). In this study, we found that prothrombin is a novel binding protein of SSL10 and SSL10 also bound to factor Xa. We demonstrated that /H9253-carboxyglutamic acid (Gla) domains of these proteins were responsible for binding and for the SSL10-inhibited blood coagulation.

EXPERIMENTAL PROCEDURES

Reagents—All of the chemical reagents were purchased from Sigma, WAKO Pure Chemicals (Osaka, Japan), and Nacalai Tesque (Kyoto, Japan). Restriction endonucleases and modifying enzymes were from TaKaRa (Osaka, Japan), Roche Applied Science, and Toyobo (Osaka, Japan). Oligonucleotides were supplied by Nihon Gene Laboratories (Sendai, Japan). Glutathione-Sepharose 4B, nickel-Sepharose 6 Fast Flow, NHS-activated Sepharose 4 Fast Flow, and benzamidine-Sepharose 4 Fast Flow were products of GE Healthcare. Human prothrombin was purchased from Calbiochem. Human factor VIIa, IXa, and Xa were products of Hematologic Technologies Inc. (Essex Junction, VT). Human IgG was obtained from serum by the combination of ammonium sulfate precipitation and affinity purification using protein G-Sepharose (GE Healthcare). PS-FIGURE 1. The interaction of SSL10 with prothrombin. A, isolation of SSL10-binding proteins from porcine plasma. Porcine plasma (15 ml) was mixed with SSL7- and SSL10-conjugated Sepharose or unconjugated Sepharose (10 /H92621 l, 50% of slurry) and incubated at room temperature for 1 h, and the resins were washed five times with wash buffer. The porcine plasma proteins bound to the resins were separated on SDS-PAGE using 10% polyacrylamide gel and stained with CBB. The protein specifically recovered by SSL10-conjugated Sepharose (80-kDa protein indicated by arrowheads) was subjected to peptide mass fingerprinting analysis. B, pull-down assay. Two microliters of SSL7- or SSL10-conjugated Sepharose (50% slurry) was incubated with a mixture of human IgG (0.067 nmol), human prothrombin (0.14 nmol), and BSA (10 /H9262g) at 4 °C for 1 h. Bound (B) and unbound (UB) fractions were analyzed by SDS-polyacrylamide gel (10%) under reducing conditions followed by CBB staining. The amount of IgG and prothrombin recovered by the resin was estimated by the densitometric analysis on the bands corresponding to them using ImageJ version 1.46r software (National Institutes of Health). C, solid phase binding assay. Human prothrombin (0–6.9 × 10⁻³ nmol) was adsorbed to a microtiter plate well at 4 °C for 16 h. After blocking (B) and unbound (UB) fractions were analyzed by SDS-polyacrylamide gel (10%) under reducing conditions followed by CBB staining. The amount of IgG and prothrombin recovered by the resin was estimated by the densitometric analysis on the bands corresponding to them using ImageJ version 1.46r software (National Institutes of Health). D, solid phase binding assay. Human prothrombin (0–6.9 × 10⁻³ nmol) was adsorbed to a microtiter plate well at 4 °C for 16 h. After blocking (B) and unbound (UB) fractions were analyzed by SDS-polyacrylamide gel (10%) under reducing conditions followed by CBB staining. The amount of IgG and prothrombin recovered by the resin was estimated by the densitometric analysis on the bands corresponding to them using ImageJ version 1.46r software (National Institutes of Health). TABLE 1 Observed mass peaks detected by the MS analysis and the corresponding amino acid sequences of porcine thrombin

| m/z | Amino acid sequencea | m/z | Amino acid sequencea |
|-----|----------------------|-----|----------------------|
| 2783.24 | KPVVPEFVKLPQETK | 2561.04 | PYSQVEVPQVFQK |
| 2342.76 | EKLVQEGNALGMNAYRY | 1774.51 | KSQPLCLCGASLSDR |
| 3203.83 | ALSVDQQMPVPLVENDC | 1500.21 | DFTADVQFPVNEK |
| 2321.82 | TVEGSDAEGLAPWSVMFFR | 1526.36 | CDACEGDSGPFVMK |
| 2144.69 | WYQMGIVGWSGWEGGDRK | 1285.02 | YVWNRENDK |
| 2159.62 | NPKSITGPPWCTYTVR | 1213.08 | ELFESYIER |
| 2985.57 | LVEECLENGAGCGLGMYVR | 1190.06 | VGFYTHVFV |
| 2991.57 | TDNMFCAGYKPEDEGR | 1149.01 | SNGEEOQWR |
| 1986.57 | NSGTRTVNVSPLLETCPYER | 898.95 | VTAEIPR |
| 1969.60 | WVLTAAYCLLPPYDK | 874.87 | VGTWGNLK |
| 1957.54 | LAVTSHGSPCLAWSSQA | 798.88 | 4YVIPRE |
| 1921.50 | QDDFNPAVLVPENFRC |  |

* GenBank™ accession number: Q19A28, PIg (prothrombin protein, Sus scrofa). Sequence coverage was 53%. The Mowse score of this analysis was 142.

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CXCRI4 and inhibits migration of T cell leukemia (12); SSL10 binds to human IgG, interferes with the interaction between IgG and complement C1q, and inhibits the classical complement activation pathway (13) and Fc receptor-mediated phagocytosis of neutrophils (14); and SSL3 binds to TLR2 (Toll-like receptor 2) and inhibits cytokine production from macrophages in response to TLR2 ligands (15, 16).
containing liposomes (phosphatidylcholine/PS/cholesterol = 5.5:1:5:3 molar ratio, 25 nmol of lipid/μl) were prepared by a dry film method as described previously (17).

Preparation of Recombinant SSLs, Coagulase, and SSL-conjugated Sepharose—Recombinant SSLs and SSL-conjugated Sepharose were prepared as described previously (15). Recombinant coagulase (residues 1–325) was produced as N-terminal glutathione S-transferase (GST) fusion protein and then excised using factor Xa. The gene for coagulase was amplified by PCR using genomic DNA of *S. aureus* (ATCC 277933) as a template and the primers 5′-GGG ATC CCC ATA GTA ACA AAG GAT TAT AGT GGG-3′ (BamHI site underlined) and 5′-GGT CGT ATA TTG TGT TGT TTC AGC TTT AC-3′ (Sall site underlined) and cloned into pGEM-T Easy (Promega, Madison, WI) for DNA sequencing. The mRNA fragment of coagulase was excised with BamHI and Sall and cloned into the expression vector pGEX-5X-1 (GE Healthcare). To remove 3 amino acid residues between the factor Xa cleavage recognition sequence and the N terminus of coagulase (Gly-Ile-Pro, which is derived from the multicloning site of pGEX-5X-1), 9 base pairs were deleted by site-directed mutagenesis using oligonucleotides 5′-GGG ATC CCC ATA GTA ACA AAG GAT TAT AGT GGG-3′ and 5′-TAC TAT ACG ACC TTC GAT CAG ATC C-3′. The *Escherichia coli* strain BL21 was transformed with the resultant construct, and recombinant protein was induced by the culture at 37 °C for 4 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako). The *E. coli*-produced GST-coagulase was lyzed by sonication, and the fusion protein was bound to glutathione-Sepharose 4B. The resin was washed three times and then equilibrated with factor Xa buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM CaCl₂, pH 6.5) and incubated with factor Xa (9.5 μg, 0.22 nmol/250 μl) for 16 h at 25 °C. The reaction mixture was recovered and passed through benzamidine-Sepharose 4 Fast Flow to remove factor Xa. Protein concentration was determined with a BCA protein assay kit (Pierce). The purities of recombinant proteins were estimated to be more than 95% by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining.

Preparation of Mouse Anti-SSL10 Serum—Mouse anti-SSL10 serum was generated by immunizing mice (ICR, male, 6W, Japan SLC, Hamamatsu, Japan) with recombinant SSL10 (50 μg/mouse) as antigen emulsified in Freund’s complete adjuvant (BD Biosciences), boosting 2 weeks later with the antigen in incomplete Freund’s adjuvant (BD Biosciences), collected by cardiac puncture under anesthesia at 2 weeks postboosting. The serum was pooled from five immunized mice. Anti-SSL10 serum did not cross-react with SSL3, SSL5, and SSL7 in immunoblot analysis.

Preparation of Porcine, Mouse, and Human Plasma—Porcine plasma (15 ml) was mixed with SSL-conjugated Sepharose (10 μl of 50% slurry) and incubated at room temperature for 1 h, and the resin was washed five times with wash buffer (25 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 1% Nonidet P-40). Proteins bound to SSL-conjugated Sepharose were eluted into 20 μl of 2X Laemml’s sample buffer (100 mM Tris-HCl, pH 6.8, 20% SDS, 20% glycerol, 0.02% bromphenol blue, 10% 2-mercaptoethanol) (19), separated on SDS-PAGE using a 10% polyacrylamide gel, and stained with CBB R250 (Merck). The protein specifically recovered by SSL-conjugated Sepharose was identified using peptide mass fingerprinting analysis as described previously (11, 13).
SSL10 Inhibits Blood Coagulation

A

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B

C

D

E

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SSL10 Inhibits Blood Coagulation

Surface Plasmon Resonance Analysis—Surface plasmon resonance analysis was performed using the ProteOn XPR36 system (Bio-Rad). Recombinant SSL10 was immobilized on a ProteOn GLC sensor chip by amine coupling according to the manufacturer’s instructions. Prothrombin or IgG was diluted with 10 mM HEPES plus 0.01% Tween 20 (pH 7.4) and injected into the chip at a flow rate of 100 μl/min. The equilibrium dissociation constants were calculated using ProteOn Manager™ software.

Examination of Anticoagulant Activity of SSL10—The effect of SSL10 on blood coagulation was examined by a plasma clotting assay. Varying amounts of SSL10 (0–10 μg (0–0.39 nmol)/test tube, dissolved in 10 μl) were added to 100 μl of citrated human plasma and incubated for 5 min at room temperature, added to 10 μl of aPTT reagent (Roche PT reagent, Roche Applied Science), and then incubated for 2 min at 37 °C. After low speed centrifugation, supernatant was transferred to a microtiter plate. The clotting reaction was started by the addition of 10 μl of 100 mM CaCl₂, and the absorbance at 415 nm every 10 s was monitored for 6 min using a microplate reader. aPTT was measured using Roche PT reagent according to the manufacturer’s protocol except for citrated plasma (0.1 ml), which was incubated with or without SSL10 (10 μg, 0.39 nmol) at 37 °C for 5 min in advance. To examine the effect of SSL10 on plasma clotting induced by factor Xa, citrated human plasma (100 μl) supplemented with PS-containing liposomes (2.5 nmol of lipid) was incubated with SSL10 (10 or 27 μg, 0.39 or 1.1 nmol) for 5 min at room temperature and transferred to the well preadded with factor Xa (0.033 μg, 7.7 × 10⁻⁵ nmol) and 100 μm CaCl₂ (10 μl), and plasma clotting was monitored as described above. To examine the effect of SSL10 on coagulase-induced clotting, the clotting reaction was started by the addition of recombinant coagulase (500 ng, 13 nmol) to the citrated plasma pretreated with SSL10.

Protease Activity of Prothrombin—The protease activity of thrombin was measured by hydrolysis of chromogenic thrombin substrate peptide. Bovine α-thrombin (Sigma) or supernatant of recalcified plasma was incubated with 0.1 mm chromogenic thrombin substrate (H-Sar-Pro-Arg-p-nitroanilide, HCL, Calbiochem) in the presence or absence of SSL10 at 25 °C in TBS. Hydrolysis of the substrate was measured by recording the absorbance at 415 nm in a microplate reader every 1 min. The concentration of hydrolyzed product was determined from a standard curve of p-nitroaniline concentration versus absorbance at 415 nm. The effect of SSL10 on conversion of prothrombin to the active form was measured by an increment of thrombin activity in recalcified plasma. Citrated plasma (0.05 ml) was preincubated for 5 min at 37 °C in the presence or absence of SSL10 (10 μg, 0.39 nmol) and incubated with or without SSL10 (10 μg, 0.39 nmol) in 50 μl of citrated plasma pretreated with SSL10.
SSL10 inhibits blood coagulation

Absence of SSL10. Activation of prothrombin was initiated by adding 0.05 ml of 25 mM CaCl₂ and terminated by adding 0.1 ml of 100 mM EDTA. The generated thrombin activity in the supernatant was measured using chromogenic thrombin peptide as described above. To analyze the effect of SSL10 on generation of staphylocoagulase by coagulase, prothrombin (1 μg, 0.014 nmol) was incubated with coagulase (0.25 μg, 6.6 × 10⁻³ nmol) in the presence or absence of SSL10 (2 μg, 0.079 nmol), and then hydrolysis of the substrate was measured by continuously recording the absorbance at 415 nm as described above. The statistical difference was assessed by Student’s t test.

RESULTS

SSL10 binds to prothrombin—We attempted to isolate novel SSL-binding proteins from porcine plasma by affinity purification using His₆-tagged SSL-conjugated Sepharose. As shown in Fig. 1A, an 80-kDa protein was specifically observed in the electrophoretic protein profile recovered by SSL10-conjugated Sepharose fraction but not by SSL7-conjugated Sepharose. SSL7-conjugated Sepharose recovered IgA (65 kDa, heavy chain of IgA in Fig. 1A) as previously reported (6). Other SSL-conjugated Sepharose failed to recover specific proteins from porcine plasma (data not shown). The 80-kDa protein recovered by SSL10-conjugated Sepharose was identified as porcine prothrombin by peptide mass fingerprint analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Mass peaks detected by the MS analysis and the Mascot search analysis are summarized in Table 1. To examine whether SSL10 recognizes human prothrombin, we performed pull-down analysis using SSL10-conjugated Sepharose and commercially available human prothrombin. SSL10- but not SSL7-conjugated Sepharose recovered human prothrombin from the mixture of human prothrombin and BSA (Fig. 1B). We previously reported that SSL10 binds to human IgG (13), and SSL10-conjugated Sepharose preferentially recovered prothrombin rather than human IgG from the mixture of two proteins; the amounts of prothrombin and IgG (H chain) recovered by SSL10-conjugated Sepharose was increased according to the amount of prothrombin immobilized on the well, and saturation of the binding was observed (Fig. 1C). In surface plasmon resonance analysis, the equilibrium dissociation constant between SSL10 and prothrombin (Kₐ = 1.36 × 10⁻⁷ M; Fig. 1D) was smaller than that between SSL10 and IgG (Kₛ = 2.51 × 10⁻⁷ M; Fig. 1E). These data indicate that SSL10 is able to bind to porcine and human prothrombin.

SSL10 binds to fragment 1 + 2 of prothrombin as well as factor Xa—To identify the region of prothrombin responsible for binding to SSL10, we performed pull-down analysis using SSL10-conjugated Sepharose and fragments of prothrombin generated by factor Xa digestion (Fig. 2A). As shown in Fig. 2B, fragment 1 + 2, which is composed of the Gla domain and kringle 1 and 2 (~27 kDa in unreduced condition) but not the
serine protease domain of thrombin (~35 kDa in unreduced condition) was recovered by SSL10-conjugated Sepharose. Interestingly, factor Xa used for the digestion also bound to SSL10-conjugated Sepharose (Fig. 2B). Factor Xa was independently co-precipitated with SSL10-conjugated Sepharose (Fig. 2B). These data indicate that SSL10 is able to bind to fragment 1 + 2 of prothrombin as well as factor Xa.

**Gla Domain Is Required for the Interaction between SSL10 and Coagulation Factors**—Because the Gla domain is shared among fragment 1 + 2 of prothrombin and factor Xa, we examined the involvement of the Gla domain in the interaction using Gla domain-less prothrombin and factor Xa prepared by limited digestion of them with α-chymotrypsin (Fig. 3A). Removal of the Gla domain completely abolished binding of prothrombin and factor Xa to SSL10-conjugated Sepharose (Fig. 3, B and C). The Gla domain of coagulation factors contains post-translationally modified γ-carboxyglutamic acids, and an anticoagulation medicine, warfarin, is known to inhibit the post-translational modification by preventing recycle of vitamin K. The recovery of prothrombin from plasma by SSL10-conjugated Sepharose was diminished by the treatment of mice with warfarin (Fig. 3D). Coagulation factors VII and IX are vitamin K-dependent Gla-containing proteins, and SSL10 was able to bind to immobilized factor VIIa and IXa in a manner similar to prothrombin and factor Xa (Fig. 3E). These results indicate that the Gla domain of the coagulation factors is responsible for their binding to SSL10, and modification of γ-carboxyglutamic acid residues in the Gla domain is essential for the interaction.

**The Interaction between SSL10 and Prothrombin Is Competed by Ca²⁺**—The Gla domain of coagulation factors is known to bind to Ca²⁺. We analyzed the effect of Ca²⁺ on the interaction of prothrombin with SSL10. The co-precipitation of prothrombin with SSL10-conjugated Sepharose was decreased in the presence of CaCl₂. The inhibitory effect of Ca²⁺ on SSL10-prothrombin interaction was dose-dependent; the amounts of prothrombin bound to the resin were decreased by 8.94, 44.4, and 56.9% in the presence of 1.25, 10, and 50 mM of Ca²⁺, respectively, by densitometric analysis (Fig. 4A). In another experiment, the interaction was decreased by 48.2% in the presence of CaCl₂ (10 mM) but not affected in the presence of EDTA (10 mM, increased by 1.3%). A similar result was obtained with the solid phase binding assay because the binding of SSL10 to immobilized prothrombin was decreased by 44.1 and 15.8% by the addition of 50 and 10 mM CaCl₂, respectively. The binding was slightly and non-significantly decreased by the physiological concentration of Ca²⁺ (Fig. 4B).

Twice the concentration of NaCl slightly inhibited the interaction, but this inhibition was significantly smaller than that by CaCl₂ (Fig. 4C). These results indicate that the interaction between SSL10 and prothrombin was competed by Ca²⁺.

**SSL10 Inhibits Plasma Clotting via Inhibiting Activation of Prothrombin but Not Thrombin Activity**—We examined the effect of SSL10 on blood coagulation. Plasma clotting after activation of the intrinsic pathway was prolonged by the addition of SSL10 (0.025–0.39 nmol) to 100 μl of plasma but not SSL7 (Fig. 5). The prolonging effect of SSL10 on plasma clotting was dose-dependent; 0.39, 0.2, and 0.098 nmol of SSL10 prolonged clotting time by 45.1, 20.5, and 7.6%, respectively. The anticoagulant activity of SSL10 was observed by a conventional aPTT method (control value was 38 s; 0.79 nmol of SSL10 prolonged clotting time by 28 s, 73.7%; 0.39 nmol of SSL10 prolonged it by 8 s, 21.1%). On the other hand, SSL10 did not inhibit hydrolysis of thrombin substrate by thrombin, and protease activities of bovine α-thrombin and supernatant of recalcified mouse plasma were not inhibited by a more than 1000-fold molar excess of SSL10 (Fig. 6, A and B). The binding of SSL10 to immobilized bovine α-thrombin used in this study was quite low (less than 10%) as compared with immobilized human prothrombin in solid phase binding analysis (data not shown).

Then we examined the temporal generation of thrombin activity in recalcified plasma in the presence or absence of SSL10, as described in the legend to Fig. 7A. The protease activity of thrombin was successively increased after the addition of Ca²⁺ to citrated plasma; however, it was strongly suppressed in the
SSL10 Inhibits Blood Coagulation

**A**

![Graph](image)

**B**

![Graph](image)

FIGURE 6. Effect of SSL10 on hydrolysis of thrombin substrate by α-thrombin. A, bovine α-thrombin (0.5 millunits/7.3 × 10^−5 nmol; Sigma) was incubated with 0.1 μM of chromogenic thrombin substrate in the presence or absence of SSL7 (0.1 nmol) or SSL10 (0.098 nmol) at 25 °C in TBS. The hydrolyzed product was measured by continuously recording the absorbance at 415 nm in a microplate reader. B, supernatant of recalcified mouse plasma (100 μL) was incubated with the thrombin substrate in the presence or absence of SSL10 (0.20 nmol) at 25 °C in TBS, and the hydrolyzed product was measured using a microplate reader. Data were shown as means ± S.D. (error bars) of triplicates.

presence of SSL10 (0.79 nmol) in 100 μL of plasma (Fig. 7, B and C). These results indicate that SSL10 inhibits blood coagulation via diminishing the generation of thrombin but not by inhibiting the protease activity of thrombin.

SSL10 Attenuates Clotting Induced by the Addition of Xa to Plasma—To examine whether SSL10 inhibits conversion of prothrombin to thrombin by factor Xa, we observed the clotting induced by the addition of factor Xa and Ca^{2+} to citrated plasma in the presence or absence of SSL10. Factor Xa (0.077 nmol added to 100 μL plasma) induced plasma clotting in the presence of Ca^{2+} and PS-containing liposome, and SSL10 (0.39 nmol) delayed the clotting by 20 s, although the delay by SSL10 was shorter than that of aPTT reagent-induced clotting (Fig. 8A). The delay time was increased by a larger amount of SSL10 (1.1 nmol) (Fig. 8B), and the effect of SSL10 on the clotting was dependent on the PS-containing liposomes (Fig. 8, B–D). The addition of phosphatidylcholine liposome did not accelerate the coagulation induced by Xa (data not shown). These results indicate that SSL10 is able to inhibit the conversion of prothrombin to thrombin by factor Xa in plasma, especially on the cell surface membrane.

**DISCUSSION**

In this study, we demonstrated that one of the SSL family exoproteins, SSL10, binds to the coagulation factors prothrombin and factor Xa and is able to inhibit blood coagulation. The physiological functions of SSL10 have been revealed (*i.e.* SSL10 binds to the Fc region of human IgG and inhibits the complement activation through the classical pathway (13) and IgG-Fcγ receptor interaction to inhibit phagocytosis (14), inhibits CXCL12-induced leukemia cell migration by blocking CXCR4 (12), and binds to phosphatidylserine and recognizes apoptotic cells (17)). Taken together, SSL10 seems to possess pleiotropic effects on host immunity and homeostasis.

We could not identify prothrombin as the SSL10-binding protein when we isolated human IgG from human serum using SSL10-conjugated Sepharose (13). That was because the concentration of IgG (7.7–20 mg/ml) was extremely higher than that of prothrombin (0.1–0.15 mg/ml) in plasma, and SSL10 binds primate IgG but not other mammal IgG (14). Therefore, we were able to isolate prothrombin as SSL10-binding protein using porcine plasma in this study. SSL10 inhibited
coagulation of human plasma, indicating that SSL10 acts as anticoagulant in the presence of IgG, and we could not detect other Gla-containing coagulation factors (factors VII, IX, and X) in the SSL10-conjugated Sepharose binding fraction (Fig. 1A), regardless of their binding ability to SSL10 (Fig. 3E). It is likely that their amount was less than that of prothrombin in plasma. In Fig. 1A, 150 kDa of protein also seemed to be specifically recovered by SSL10-conjugated Sepharose; however, its amount was too small to identify the molecule.

SSL10 binds to both prothrombin and factor Xa via their N-terminal Gla domain. Thrombin, the Gla domain-less form of prothrombin, and factor Xa did not bind to SSL10 (Figs. 2B and 3E), and SSL10 did not affect the protease activity of thrombin (Fig. 6A). This is reasonable because all of them lose the Gla domain during activation or digestion by protease. SSL10 binds to prothrombin of various species because the Gla domain of prothrombin is highly conserved among species (human versus pig (78%) and human versus mouse (88%)).

**FIGURE 7. The effect of SSL10 on the generation of thrombin activity in recalcified plasma.** A, scheme for analyzing the effect of SSL10 on the generation of thrombin activity in recalcified plasma. B, CaCl₂ (25 mM, 50 μl) was added to mouse citrated plasma (50 μl) preincubated with or without SSL10 (0.79 nmol). After incubation for the indicated time, activation of prothrombin was terminated by the addition of EDTA (100 mM, 100 μl). The generated thrombin activity in the supernatant was measured using chromogenic thrombin peptide as described under “Experimental Procedures.” B, time course graph for hydrolysis of thrombin substrate by recalcified plasma prepared as shown in A. C, graph for velocity of the hydrolysis of thrombin substrate by recalcified plasma.
Fig. 3, B and C, we could not detect digested Gla domain, because it was too small (the fragment of prothrombin is estimated to be 9 kDa). The viral protein adenovirus serotype 5 hexon protein is reported to bind to the Gla domain and is responsible for the entry of this virus into hepatocytes (20). To our knowledge, this is the first report indicating that the bacterial exoprotein binds to the Gla domain of coagulation factors. There is no homology among them; therefore, each protein binds to the Gla domain of coagulation factors via a distinct mechanism.

Prothrombin existing in plasma from warfarin-treated mice failed to bind to SSL10 (Fig. 3D), and the interaction of SSL10 with prothrombin was attenuated by the excess concentration of Ca\(^{2+}\) (Fig. 4, A–C), suggesting that SSL10 recognizes \(\gamma\)-carboxyglutamic acid in the Gla domain. Because the inhibition by twice the concentration of NaCl was significantly smaller than that by CaCl\(_2\) (Fig. 4C), the contribution of the ionic strength of CaCl\(_2\) to the inhibition was small. The competitive effect of Ca\(^{2+}\) on SSL10-prothrombin interaction was limited at 1.25 mM of Ca\(^{2+}\); therefore, the interaction between SSL10 and prothrombin will be significant in physiological conditions. We previously reported that SSL10 binds to PS (17). Recombinant GST-annexin V, which interacts with PS, did not bind to SSL10 (data not shown), suggesting that the PS-binding feature does not account for the binding of SSL10 to prothrombin.

The physiological importance of the Gla domain is well established; the domain binds to Ca\(^{2+}\) and is involved in the assembly of coagulation factors on the surface of activated platelets, which is essential for effective activation of prothrombin. In this study, SSL10 delayed blood coagulation induced by the addition of Ca\(^{2+}\) (Fig. 5), and SSL10 abrogated the generation of thrombin activity in recalcified plasma (Fig. 7, B and C), suggesting that SSL10 inhibits blood coagulation via interrupting the activation of prothrombin, the penultimate crucial step of the coagulation cascade. SSL10 inhibited generation of thrombin activity in plasma by the addition of factor Xa and Ca\(^{2+}\), and the inhibitory effect of SSL10 was dependent on the amount of PS-containing liposomes added to the plasma (Fig. 8). In this experiment, the addition of PS-liposome would increase the site of assembly for coagulation factors, and SSL10 may inhibit the assembly of them. Because SSL10 interacted with all of the Gla-containing coagulation factors (prothrombin and factors VII, IX, and X), it is likely that SSL10 also interferes with their functions in coagulation cascade. Indeed, the inhibitory effect of SSL10 on clotting induced by the addition of factor Xa (Fig. 8) was weaker than that on clotting induced by aPTT reagent (Fig. 5). We previously reported that SSL10 is able to bind phosphatidylserine (17). It is possible that SSL10 bridges prothrombin with phospholipid in the absence of Ca\(^{2+}\), but the present data exclude this possibility. The significance of SSL10-phospholipid interaction awaits further study.

*S. aureus*, but not other weakly pathogenic staphylococci, produces the extracellular protein coagulase, which binds to prothrombin and forms an enzymatically active complex called

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**FIGURE 8. Effect of SSL10 on plasma clotting induced by factor Xa.** SSL10 (0.39 or 1.1 nmol) was added to citrated human plasma (100 μl) supplemented with PS-containing liposomes (2.5–7.5 nmol of lipid), the mixture was incubated for 5 min at room temperature and transferred to the well preadded with factor Xa (7.7 × 10\(^{-4}\) nmol) and 100 mM CaCl\(_2\) (10 μl), and plasma clotting was monitored as described under “Experimental Procedures.” A, SSL10 (0.39 nmol) was added to 100 μl of plasma supplemented with 0.25 nmol of PS-containing liposome. B–D, SSL10 (1.06 nmol) was added to 100 μl of plasma supplemented with 0.25 or 0.75 nmol of PS-containing liposome (B or C, respectively) or without liposome (D). The dotted lines in the graphs show the time point of 50% clotting, and the double-headed arrows show the difference of the half-clotting time between control and SSL10.
staphylothrombin (21). Coagulase is thought to be responsible for the virulence of \textit{S. aureus} (22). SSL10 did not inhibit the proteolytic activity of staphylothrombin, mildly attenuated the formation of staphylothrombin, and inhibited plasma clotting induced by coagulase. However, the anti-coagulant effect was slight as compared with that on the intrinsic pathway. These results suggest that the inhibitory effect of SSL10 on staphylococcal coagulase is limited. Probably, SSL10 is able to disturb physiological blood coagulation with minimal effect on coagulase. \textit{S. aureus} produces a series of proteins that affect coagulation and fibrinolysis (i.e. coagulase, von Willebrand factor-binding protein (23), staphylokinase (24), and SSL10). The regulation of or interference with coagulation and fibrinolysis would be beneficial for \textit{S. aureus}.

The target proteins of the SSL family found so far are mostly immune molecules, suggesting that the SSL family contributes to the immune evasion of \textit{S. aureus}. The present findings suggest the idea that the SSL family is a wide spectrum disturbing factor against not only host immunity but also homeostasis. Fourteen SSLs share low amino acid homology; therefore, each SSL would have a corresponding binding partner, and the functions of most SSL proteins remain uncharacterized. Moreover, several SSLs have multiple binding partners (e.g. SSL5 (binds to PSGL-1), chemotactant receptors, matrix metalloproteinase-9, GPIIbα, and GPIIIb/IIa), SSL7 (IgA and C5) and SSL10 (CXCR4, IgG, phosphatidylycerine, and prothrombin)). The identification of all of the SSL target molecules will contribute to the understanding of the virulence and pathogenesis of \textit{S. aureus}.

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