A Cysteine-rich Protein from an Arthropod Stabilizes Clotting Mesh and Immobilizes Bacteria at Injury Sites*

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Hemolymph coagulation in arthropods plays key roles in host defense, including sealing wounds to staunch bleeding and immobilizing invading microorganisms. We have previously reported that horseshoe crab transglutaminase (TGase) promotes cross-linking of a clotting protein (coagulin) with hemocyte-derived proteins (proxins), resulting in the formation of stable coagulin fibrils. Here we show that TGase also cross-links proxins to another hemocyte-derived protein named stablin. Stablin is a cysteine-rich protein of 131 residues. Surface plasmon resonance analysis revealed the specific interaction of stablin with proxin-1 at \( K_d = 4.0 \times 10^{-8} \text{ M} \). Stablin was predominantly localized in the large granules of hemocytes and secreted by lipopolysaccharide-induced exocytosis. Interestingly, stablin bound to chitin at \( K_d = 1.5 \times 10^{-8} \text{ M} \), as determined by using a quartz-crystal microbalance. Stablin also interacted with lipopolysaccharides and lipoteichoic acids and exhibited bacterial agglutinating activity against Gram-positive and -negative bacteria. Immunostaining showed that stablin is co-localized with coagulin in the clotting fibrils that effectively trap bacteria. Moreover, an anti-stablin antibody strongly inhibited the proper formation of the clotting fibrils. These data suggest that stablin promotes the formation of the clotting mesh and the immobilization of invading microbes at injury sites. In arthropods, the TGase-mediated cross-linking may play an important role in the initial stage of host defense, wound closure, and healing, as in the case of mammals.

In arthropods, hemolymph clot is an extracellular matrix established at sites of damage to staunch bleeding and functions as an important element of the innate immune system through its ability to entrap and immobilize bacteria that have entered the body via wounds (1). In mammals blood coagulation is based on the proteolytically induced polymerization of fibrinogen (2). Initially, fibrin monomers non-covalently interact with each other, and the resulting homopolymers are further stabilized by the plasma transglutaminase (TGase) through the intermolecular cross-linking of \( \epsilon -(\gamma -\text{glutamyl) lysine bonds (3). In crustaceans, although no proteolytic cascades are involved in the hemolymph coagulation, the coagulation also depends on the TGase-mediated cross-linking of specific plasma-clotting proteins (1, 4). Insect hemolymph coagulation requires close interaction between plasma components and cellular components derived from hemocytes, resulting in the precipitation of an insoluble matrix (5). This precipitation leads to a soft clot, which is hardened through the activity of cross-linking enzymes. Recently, a proteomic study of Drosophila larval hemolymph before and after clotting has led to the isolation of several candidates for clotting factors, which include proteins with similarity to prophenoloxidase-activating proteases, two phenoloxidases, and substrates for the TGase (6). On the other hand, hemocoept, one of the most abundant hemolymph proteins in Drosophila, has been isolated and characterized as a coagulation-related factor (7).

In horseshoe crabs, granular hemocytes comprise 99% of all hemocytes and are involved in the storage and release of defense molecules, including serine protease zymogens, the clot-able protein coagulogen, protease inhibitors, antimicrobial peptides, and lectins (8, 9). In response to the stimulation from bacterial lipopolysaccharides (LPS), the defense molecules stored in granules are secreted by hemocyte exocytosis (8, 10, 11). The coagulation cascade of these zymogens is triggered by LPS or \( \beta-1,3-\text{glucans}, \) which finally leads to the conversion of coagulogen into coagulin, resulting in noncovalent coagulin homopolymers through head-to-tail interaction (12, 13).

Moreover, LPS has been shown to induce the secretion of horseshoe crab TGase from the hemocyte cytosol through an unknown mechanism (14). Horseshoe crab TGase shows a significant sequence similarity with the mammalian TGase members (15, 16): human keratinocyte TGase (37.6% identity), human factor XIIIa subunit (34.7%), and guinea pig liver TGase (32.7%). Although horseshoe crab TGase neither catalyzes monodansylcadaverine (DCA) incorporation into coagulin nor cross-links coagulin intermolecularly, TGase cross-links coagulin with the hemocyte-derived proline-rich proteins known as proxins, resulting in more stably clotting fibrils (14, 17). In the American horseshoe crab Limulus polyphemus, at least three hemolymph proteins, i.e. \( \alpha_2 \)-macroglobulin, C-reactive proteins, and hemocyanin, are involved in the formation of the...
clotting fibrils (18). The clotting mesh cross-linked by TGase may function to seal the wound to stop bleeding, serve as a barrier to the entry of microbes into the interior of the animal via the wound, and operate as a transient extracellular matrix for the migration of wound-healing epithelial cells. In this study we identified a TGase substrate of 15 kDa, named stablin, as a shorthand for clot-stabilizing protein, and demonstrated that stablin is an essential component of the clotting mesh and, thus, functions in the forefront of innate immunity.

EXPERIMENTAL PROCEDURES

Materials—Hemocyte debris from the Japanese horseshoe crab *Tachypleus tridentatus* was prepared as described previously (19). LPS from *Escherichia coli* O111:B4, *E. coli* O55:B5, and *Salmonella minnesota* RS595 and lipid A from *E. coli* K12 were from List Biological Laboratories, Inc. (Campbell, CA). LPS from *E. coli* O128:B12, *E. coli* O127:B8, and *E. coli* O26:B6, *Vibrio cholerae* Inaba 569B, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Shigella flexneri* 1A, and lipoteichoic acids from *Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus sanguis*, and *Bacillus subtilis* were from Sigma. LPS from *V. cholerae* O22 and *V. cholerae* O139 were kindly provided by Drs. S. Kondo and K. Hisatsune of Josai University, Japan. The sources of the other materials used were as follows: Sephadex G-50 fine, S-Sepharose fast flow, Sepharose CL-6B, and DEAE-Sepharose CL-6B were from Amersham Biosciences, chitin was from Seikagaku Corp., Tokyo, lysyl endopeptidase was from Wako Pure Chemical Industries, Ltd., Tokyo, and trypsin was from Worthington Biochemical Co., Freehold, NJ.

Expression of Horseshoe Crab TGase in Insect Cells—A DNA fragment encoding TGase (pTG74) (16) followed by a stop codon was created by PCR and subcloned into the NotI and EcoRI sites of the expression vector pFastBac™1 (Invitrogen). The recombinant plasmid was transformed into DH10Bac™ competent cells (Invitrogen) that contained the bacmid with a mini-att Tn7 target site and the helper plasmid according to the manufacturer’s specifications. The mini-Tn7™ element on the pFastBac™ donor plasmid can transpose to the mini-att Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. The transposed bacmid was transfected into Sf9 insect cells with Cellfectin™ transfection reagent (Invitrogen). The resultant baculovirus pools were collected from the cell culture medium at 72 h post-transfection. The insect cells were infected with the recombinant virus and harvested 72 h after the infection. The cells were homogenized in 50 mM Tris acetate, pH 7.5, containing 1 mM EDTA and 1% Nonidet® P-40. The homogenized cell lysate was centrifuged, and the resulting supernatant was applied to a Sepharose CL-6B column. The TGase activity of each fraction was determined by DCA incorporation into proxins (14).

Incorporation of DCA into Hemocyte-derived Proteins by TGase—Hemocyte extract was incubated with TGase in 50 mM Tris acetate, pH 7.5, containing 10 mM CaCl₂ and 0.5 mM DCA at 37 °C for 1 h. The aliquot was subjected to SDS-PAGE according to Laemmli (20). The fluorescence-labeled proteins were visualized by a trans-illuminator.

Purification of a TGase Substrate of 15 kDa—The hemocyte debris (77.3 g, wet weight) was extracted twice by homogenizing with 200 ml of 30% acetic acid, and the supernatant obtained by centrifugation at 14,000 rpm for 15 min was lyophilized. The dried material was dissolved in 100 ml of 10% acetic acid then applied to a Sephadex G-50 column (3.6 × 110 cm) equilibrated with 10% acetic acid (Fig. 1A). SDS-PAGE in a 15% gel indicated the presence of the 15-kDa protein in fractions 60–70 (data not shown). These fractions were collected, lyophilized, and then applied to an S-Sepharose fast flow column (2 × 32 cm) equilibrated with 20 mM Tris·HCl, pH 8.0, containing 0.1 M NaCl. After washing with the equilibration buffer, proteins were eluted with a linear NaCl gradient of 0.1–0.4 M in the same buffer (Fig. 1B). SDS-PAGE in a 15% gel indicated the presence of the 15-kDa protein in fractions 95–99 (data not shown). These fractions were collected and further separated into 3 peaks by a phenyl-5PW reverse-phase HPLC (rpHPLC) column (4.6 × 75 mm) (Fig. 1C). The partial NH₂-terminal sequence analysis revealed that the protein at peak 1 contained the previously characterized protease inhibitor of 7 kDa, a Kunitz-type protease inhibitor (21). The 15-kDa protein was eluted at peak 2. The extinction coefficient of the 15-kDa protein at 280 nm for 1% solution in water was calculated by using the data from the amino acid analysis, and the value of 11.8 was used for subsequent determinations of protein concentrations. The final yield of the 15-kDa protein from 100 g of hemocyte debris was about 4.0 mg.

Proteolytic Digestion—Protein samples were digested with lysyl endopeptidase (enzyme/substrate = 1/50, w/w) in 0.1 M NH₄HCO₃ containing 2 M urea at 37 °C for 20 h. The resulting peptides were separated by rpHPLC using a Cosmosil 5C₁₈–MS column (2.0 × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan). Peptides were eluted from the column with a linear gradient of 8–48% acetonitrile in 0.1% trifluoroacetic acid for 65 min at a flow rate of 0.2 ml/min. The effluent was monitored at 210 nm.

Amino Acid Composition and Sequence Analyses—Amino acid analysis was performed by using an AccQ-Tag system (Waters Corp., Milford, MA). Amino acid sequence analysis was carried out using an Applied Biosystems 491 protein sequencer. The protein concentration for determining the extinction coefficient of the 15-kDa protein was calculated from the amino acid mass at A₂₈₀. An internal standard, norleucine, was added to the protein hydrolysates to allow for the collection of losses.

Isolation of cDNA Clones of the 15-kDa Protein—The degenerate nucleotide sequences of primers used for reverse transcription-PCR were based on the NH₂-terminal sequence (FIKQTK) and that of the peptide derived from lysyl endopeptidase digestion (NQQQT). Sense and antisense nucleotides were synthesized with an EcoRI site at the 5’ end. The PCR reactions were carried out using a reaction mixture containing cDNA template (corresponding to 1 μg of poly(A)⁺ RNA) and 20 pmol of each primer in a Takara PCR thermal cycler (Takara Co., Ltd., Tokyo). The PCR product was treated with EcoRI and purified using agarose gel electrophoresis. The fragment of interest was then ligated into a plasmid Bluescript II SK⁺ (Stratagene, La Jolla, CA) for a sequence analysis as described by Sambrook et al. (22). The DNA sequence of the insert was ana-
lyzed, and its deduced amino acid sequence was found to be identical to the determined partial amino acid sequences of the NH₂-terminal region and the peptides derived from lysyl endopeptidase digestion of the 15-kDa protein.

**RACE—**5' or 3'-RACE analysis was carried out using a SMART™ RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, CA). This technique involved the incorporation of a “Smart Oligo” onto the 5’ end of the reverse-transcribed cDNA for the 5’-RACE analysis. For the 3’-RACE analysis, the Smart Oligo was attached to an oligo(dt) primer to yield cDNA that had a complete 3’-untranslated region, a poly-A tail, and the Smart Oligo sequence extension. The 5’ and 3’ ends of the 15-kDa protein were amplified using primers designed for RACE PCR, according to the manufacturer’s instruction manual.

**Inhibitory Effect of the 15-kDa Protein on Trypsin—**The inhibitory activities of the 15-kDa protein and the Kunitz-type protease inhibitor toward trypsin were examined as follows; trypsin (1.3 nM) was preincubated with various concentrations of the inhibitor in a total volume of 180 µl of 50 mM Tris-HCl buffer, pH 8.0, containing bovine serum albumin (0.1 mg/ml) at 37 °C. After 5 min of incubation, 20 µl of a fluorogenic peptide substrate, N-tetra-butoxycarbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide (final 0.1 mM), was added to the mixture. After 5 min at 37 °C, the reaction was stopped with 0.8 ml of 5% (v/v) acetic acid. The amount of 7-amino-4-methylcoumarin liberated from the substrates was estimated by fluorescence measurement with excitation at 380 nm and emission at 440 nm using a Hitachi fluorescence spectrophotometer 650-10S.

**Overexpression of Proxin-1 in E. coli—**To construct an expression vector for proxin-1, a DNA fragment encoding the full-length of proxin-1 (14) followed by a stop codon was created by PCR and subcloned into the Ncol and Xhol sites of the expression vector pET-28a (EMD Biosciences, Inc., San Diego, CA). The construct was verified by sequencing. Proxin-1 was expressed in the E. coli strain BL21 (DE3)/pLysS. The cells were homogenized in 50 mM Tris acetate, pH 7.5, containing 1 mM EDTA and 1% Nonidet® P-40. The homogenized cell lysate was centrifuged, and the resulting supernatant was applied to a Sepharose CL-6B column followed by a DEAE-Sepharose CL-6B column.

**Preparation of Polyclonal Antibodies against the 15-kDa Protein (Stablin)—**The immunization was carried out at Asahi Techno Glass Corp., Chiba, Japan. An antisera against stablin was raised in a rabbit as described previously (23). A polyclonal antibody against stablin was purified from the rabbit antisera with an Ampure PA kit (Amersham Biosciences). Cross-linking of Stablin with Proxin-1—Stablin (5 µg) was incubated with proxin-1 (5 µg) in the presence of TGase (0.2 µg) in 50 mM Tris acetate, pH 7.5, containing 10 mM CaCl₂ (25 µl) at 37 °C for 1 h. An aliquot of the reaction mixture was subjected to SDS-PAGE and stained with Cooamassie Brilliant Blue R-250 or transferred to nitrocellulose membrane followed by Western blotting using the anti-stablin antibody (Amersham Biosciences).

**Surface Plasmon Resonance Analysis—**Proxin-1 (50 µg/ml in 10 mM sodium acetate, pH 6.0) was immobilized on a sensor chip CM5 of the BIAcore 1000 system (Biacore, Uppsala, Sweden) according to the manufacturer’s specifications. After washing the proxin-1-immobilized sensor chip with 10 mM HCl followed by 10 mM NaOH, stablin was injected at 10 µl/min in 10 mM Hepes, pH 7.0, containing 0.15 mM NaCl, and 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 interaction obtained using various concentrations of stablin (80–100 nM) were analyzed using the software provided with the instrument.

**Western Blotting—**For Western blotting, the proteins were transferred to nitrocellulose membranes and then treated with anti-stablin antibody, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, and visualized using a Chemiluminescent One Kit (Nacalai Tesque Inc., Kyoto, Japan). Total concentrations of extracted proteins with 2% SDS were determined by using a Micro BCA™ Protein Assay reagent kit (Pierce), and 10 µg of protein of each tissue extract was applied to SDS-PAGE.

**Binding Analysis by 27-MHz Quartz-crystal Microbalances—**The interaction of stablin with chitin or LPS from S. minnesota R595 was examined using 27-MHz quartz-crystal microbalances (Initium Co., Tokyo, Japan). Chitin or LPS (5 µg) in water was immobilized onto a 27-MHz electrode. The electrode was dried at room temperature, washed with water several times to remove the excess chitin, soaked in 10 mM Hepes, pH 7.0, containing 0.15 mM NaCl and 1 mg/ml of bovine serum albumin (8 µl), and monitored continuously for frequency changes at 25 °C. The frequency changes in response to the various concentrations (2–64 nM) of stablin were assessed. The dissociation constant (Kₖ) of stablin against chitin or LPS was determined by the published method (25).

**Bacteria Agglutinating and Antimicrobial Activities—**Bacterial agglutinating activity (26) and antimicrobial activity (27) were determined as described earlier using E. coli K12. Minimum agglutinating concentrations of stablin were determined against various bacteria or fungi in 10 mM Hepes, pH 7.5, containing 50 mM NaCl. For screening of inhibitors of agglutination, carbohydrates, LPS, or lipoteichoic acids in the same buffer were incubated with stablin before adding E. coli K12.

**Immunofluorescence Microscopy—**One milliliter of T. tridentatus hemolymph was collected into 50 ml of pyrogen-free 10 mM HEPES, pH 7.0, containing 0.5 mM NaCl, and the diluted hemolymph was plated on coverslips (10). After a 20-min incubation, the attached hemocytes were incubated with 1 µg/ml LPS or E. coli expressing enhanced green fluorescence protein for 1 h and then fixed with 3.7% formaldehyde (11). The hemocytes were washed twice with phosphate-buffered saline and blocked with 5% bovine calf serum. Then the hemocytes were incubated for 1 h with anti-coagulogen monoclonal antibody and the anti-stablin polyclonal antibody. For detection, Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), rhodamine-conjugated swine anti-rabbit immunoglobulins (DACO, Glostrup, Denmark), and Alexa Fluor® 488 goat anti-mouse immunoglobulins (Molecular Probes, Eugene, OR) were used. Hemocytes were imaged with an Olympus BX-FLA fluorescence microscope (Tokyo, Japan).
RESULTS

Purification of a TGase Substrate and Its Nucleotide Sequence—
To identify potential proteins involved in the TGase-mediated hemolymph clot, hemocyte extract was incubated with TGase in the presence of DCA and subjected to SDS-PAGE (data not shown). A DCA-labeled protein with an apparent molecular mass of 15 kDa was observed accompanied with proxins and the 8.6-kDa protein, i.e. the TGase substrates already characterized (15, 17). The 15-kDa protein was then purified from hemocytes as described under “Experimental Procedures.” The purified 15-kDa protein had a single band on SDS-PAGE (Fig. 1C) and was labeled with DCA by TGase, concomitant with the cross-linked dimer, indicating a protein substrate for TGase (Fig. 1D).

A full-length cDNA of the 15-kDa protein contained a 595-nucleotide including a 30-base poly(A) tail (Fig. 2A). The open reading frame encoded a 20-amino acid signal peptide and 131-amino acid mature protein. Interestingly, homology search showed a low sequence similarity between the 15-kDa protein and keratin-related proteins (Fig. 2B): Caenorhabditis elegans F46E10.11 (28) (16.7% identity), keratin-associated protein 12.4 (29) (15.4%), keratin-associated protein 4.4 (30) (14.9%), and mouse keratin (31) (14.8%). The keratin-associated proteins, which constitute the matrix of the keratin composite of wool, are a large group of possibly up to 100 different proteins. They are divided into three main families based on their amino acid composition: ultrahigh sulfur keratin-associated proteins (>30% Cys content), high sulfur keratin-associated proteins (16–30% Cys content) (29), and high Gly/Tyr keratin-associated proteins (35–60% Gly and Tyr content) (32). Among these three types, the high sulfur keratin-associated proteins possess Cys clusters such as Cys-Cys-Val-Pro and Cys-Cys-Gln-Pro (Fig. 2B). The 15-kDa protein also contained a high Cys content (12.2%) that included Cys clusters such as Cys-Cys-Val-Pro and Cys-Cys-Ala-Thr (Fig. 2, A and B).

The 15-kDa protein was revealed to be a cysteine-rich protein (16 Cys residues), and its NH₂-terminal sequence was identical to the NH₂-terminal sequence of L2 up to the first 13 residues, which is a previously identified protein stored in the large granules of hemocytes and secreted by LPS-induced hemocyte exocytosis (33). We named the 15-kDa protein stablin, as shorthand for clot-stabilizing protein, as described later. As expected, stablin was predominantly localized in hemocytes (Fig. 3). Moreover, stablin showed significant sequence similarity with the partial NH₂-terminal sequence of a new type of trypsin inhibitor previously identified in hemocytes of the American horseshoe crab L. polyphemus, with an apparent molecular mass of 15 kDa on SDS-PAGE (34); the reported

![FIGURE 1. Elution profiles for the 15-kDa protein from a Sephadex G-50 column, an S-Sepharose column, and an rpHPLC column. A, gel filtration of the acid extract obtained from the hemocyte debris on a Sephadex G-50 column. Fractions indicated by a solid bar were collected. B, ion exchange column chromatography on an S-Sepharose fast flow column. Fractions indicated by a solid bar were collected. A broken line indicates the concentration of NaCl. C, separation of Kunitz-type protease inhibitor and the 15-kDa protein by rpHPLC. The 15-kDa protein was applied to rpHPLC and eluted at a flow rate of 0.2 ml/min with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. SDS-PAGE of the purified 15-kDa protein (peak 2) was performed under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250. D, the purified 15-kDa protein was subjected to SDS-PAGE after incubation with DCA in the presence of TGase at 37 °C for 60 min. Proteins labeled with DCA on gels were detected by UV illumination.](image-url)
NH₂-terminal sequence of the *Limulus* trypsin inhibitor is VSPPFIKQTGLFXXXFLGXSS, which is identical to stablin up to 10 residues. To test whether stablin inhibits trypsin, stablin at various concentrations was preincubated with trypsin followed by incubation with a fluorogenic peptide substrate for trypsin. Stablin did not inhibit the amidase activity of trypsin at a 10 M excess of trypsin.

During the purification of stablin, a protease inhibitor was co-purified after gel filtration and ion-exchange column chromatography and finally separated from stablin by rpHPLC (peak 1 in Fig. 1C), which was identical to the previously identified Kunitz-type protease inhibitor with Mᵣ = 6824 (21). The purified Kunitz-type inhibitor (peak 1 in Fig. 1C) strongly inhibited the tryptic activity and completely inhibited at a 5 M excess of trypsin under the same conditions. The reported inhibitory characteristics of the 15-kDa protein of *L. polyphemus* are quite similar to those of the Kunitz-type inhibitor of *T. tridentatus* (21, 34). Therefore, the inhibitory activity of the 15-kDa protein of *L. polyphemus* is possibly due to the contamination of a Kunitz-type protease inhibitor, and consequently, the 15-kDa protein of *L. polyphemus* must be a stablin ortholog, not a new type of trypsin inhibitor.

**TGase-mediated Cross-linking of Stablin with Proxin-1**—Stablin was mixed with proxin-1 or coagulin in the presence of TGase. Coagulin was not cross-linked with stablin by TGase. In contrast, proxin-1 was cross-linked with stablin, resulting in the complexes on SDS-PAGE, stablin-proxin-1, and stablin dimer-proxin-1 (Fig. 4A), which were confirmed by Western blotting using anti-stablin antibody (Fig. 4B).

We determined the binding parameters of stablin with proxin-1 by measuring surface plasmon resonance. Analysis of the association and dissociation phases of the sensorgrams, passing stablin at various concentrations over proxin-1 im-

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**FIGURE 2.** Nucleotide sequence of stablin and alignments of the amino acid sequence with various kinds of keratin. A, a nucleotide sequence of stablin. Single underlining represents the amino acid sequences determined by peptide sequencing, and the stars represent the stop signal. B, the conserved Cys residues are indicated by dots. a, stablin; b, *C. elegans* F46E 10.11; c, keratin associated protein 12.4; d, keratin associated protein 4.4; e, mouse keratin. Several unique cysteine-rich motifs are underlined.
A Cysteine-rich Protein in Horseshoe Crabs

Stablin

FIGURE 3. Tissue-specific localization of stablin. The tissue-specific localization of stablin was investigated by Western blotting using anti-stablin polyclonal antibody. Lane 1, epidermis; lane 2, hemocytes; lane 3, heart; lane 4, stomach; lane 5, intestine; lane 6, hepatopancreas; lane 7, skeletal muscle; lane 8, nerve; lane 9, plasma. All the tissues examined were from the horseshoe crab T. tridentatus.

Cross-linking of stablin with proxin-1 by TGase. A, stablin was incubated with TGase in the presence of proxin-1 (lane 2). As a negative control, stablin (lane 1) or proxin-1 (lane 3) was incubated with TGase. Aliquots were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. B, after SDS-PAGE for the same samples as shown in Fig. 4A, the proteins were detected by Western blotting using anti-stablin antibody. C, association and dissociation of stablin with the immobilized proxin-1. Sensorgrams for the binding of stablin to the immobilized proxin-1 are shown at 80 and 100 nM concentrations of stablin. The fitted lines are superimposed onto the analyzed data points (×) of the sensorgrams. RU, resonance units.

FIGURE 4.

Trimer- and tetramerized stablin from the horseshoe crab T. tridentatus were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The mobilities of these trimers and tetramers were shifted to the dimer size on the reducing gel, suggesting that intermolecular disulfide bridges of stablin could be involved in the trimer or tetramer formation. Mammalian tissue-type TGase has been reported to function as a protein disulfide isomerase (35, 36), suggesting that the cross-linking reaction by the TGase-mediated intermolecular disulfide bridges of stablin may contribute to the formation of coagulin mesh.

Involvement of Stablin in the Formation of Coagulin Mesh and Bacterial Immobilization—Coagulin fibrils are stabilized by proxins through TGase-mediated protein cross-linking (14). The present data showed that stablin interacts with proxin-1 and forms cross-linked oligomers by TGase (Fig. 4). To examine whether stablin is involved in the components of the clotting fibrils, horseshoe crab hemocytes were treated with LPS. Immunostaining of the resulting cross-linked clotting mesh with the specific antibodies against coagulogen and stablin demonstrated that stablin is co-localized with coagulin fibrils (Figs. 5, A and B). Stablin showed the bacterial agglutinating activity (Table 1), which possibly contributes to the bacterial immobilizing activity of the coagulin mesh. Hemocytes were incubated with enhanced green fluorescence protein-expressing E. coli to induce the TGase-mediated coagulin mesh. The bacteria were trapped almost completely by the clotting mesh (Figs. 5, C and D). To confirm that stablin is involved in the bacterial agglutination activity of stablin (Table 1).

Table 1

| Bacteria          | Stablin* (µg/ml) |
|-------------------|------------------|
| E. coli strain B   | 6.3              |
| E. coli C600      | 12.5             |
| E. coli K12       | 3.1              |
| E. coli kp        | 6.3              |
| E. hirae          | 12.5             |
| S. aureus 209P    | 25.0             |

* Minimum agglutinating concentrations.

Mammalian tissue-type TGase has been reported to function as a protein disulfide isomerase (35, 36), suggesting that the cross-linking reaction by the TGase-mediated intermolecular disulfide bridges of stablin may contribute to the formation of coagulin mesh.
Inhibition of bacterial agglutinating activity of stablin by LPS and lipoteichoic acid

| LPS or lipoteichoic acid | Inhibitory concentrationsa |
|-------------------------|---------------------------|
|                         | µg/ml                     |
| LPS                     |                           |
| *E. coli* O111: B4 (S)  | 12.5                      |
| *E. coli* O55: B5 (S)   | 12.5                      |
| *E. coli* O128: B12 (S) | 12.5                      |
| *E. coli* O127: B8 (S)  | 12.5                      |
| *E. coli* O26: B6 (S)   | 25.0                      |
| *E. coli* K12 (Lipid A) | 100                       |
| *S. minnesota* R595 (R) | 12.5                      |
| *V. cholerae* O122 (R)  | 6.3                       |
| *V. cholerae* O139 (S)  | 6.3                       |
| *V. cholerae* Inaba 569B (S) | 25.0                  |
| *K. pneumoniae* (S)     | 100                       |
| *P. aeruginosa* 10 (S)  | 6.3                       |
| *S. flexneri* 1A (S)    | 6.3                       |

| Lipoteichoic acid        |                     |
|--------------------------|---------------------|
| *S. aureus*              | 3.1                 |
| *S. faecalis*            | 6.3                 |
| *S. mutans*              | 25.0                |
| *S. pyogenes*            | 6.3                 |
| *S. sanguis*             | 12.5                |
| *B. subtilis*            | 12.5                |

a Minimum concentrations required for inhibition of the agglutinating activity (stablin = 6.3 µg/ml) against *E. coli* K12.

The inhibitory effects of bacterial cell wall components on agglutinating activity were examined. LPS from various Gram-negative bacteria and lipoteichoic acid, a cell wall component of Gram-positive bacteria, inhibited the bacteria agglutinating activity of stablin (Table 2). As an additional quantitative analysis, we further examined the binding of stablin to the re-type LPS from *S. minnesota* R595 using the quartz-crystal microbalance. The dissociation constant of stablin against the Re-type LPS was $K_d = 1.2 \times 10^{-8} \text{ M}$.

**DISCUSSION**

Arthropods must have efficient clotting systems in order to seal wounds and staunch bleeding as well as to prevent pathogens from invading into the hemocoel at the injured cuticle. A recent study on the *in vivo* effects of hemolymph coagulation in the American cockroach *Periplaneta americana* has demonstrated that clotting localizes immune effectors in the vicinity of the injured cuticle, which restricts the spread of invasive particles across the hemocoel, and is greater when wounding is triggered by non-self pathogens such as LPS (39). The polymerization of coagulin proceeds through the head-to-tail interaction to form non-covalent coagulin polymers, and no TGase-mediated cross-linking occurs between coagulin molecules (13). Previously, we have identified proxins that are cross-linked with coagulin by TGase to form the stable coagulin fibrils (14, 17). Both coagulogen and proxins have no chitin binding activity, and thus, the involvement of a chitin-binding protein(s) in the coagulin mesh formation is thought to be required for effective sealing and bacterial immobilization at the injured cuticle. Expectedly, stablin agglutinated several kinds of Gram-negative and Gram-positive bacteria (Table 1). This broad binding specificity of stablin was similar to that of horseshoe crab lectins, such as tachylcin-1 (26) and tachylcin-5 (40), and may be very important for primary pattern recognition against invading microbes.

In the American horseshoe crab *L. polyphemus*, microbes entrapped in the clotting mesh survive and proliferate in artificial seawater, whereas the immobilized microbes are killed in the presence of plasma; neither clot nor plasma alone kill the bacteria, indicating the importance of synergy between the two for effective killing of pathogens (41).

In mammals the cornified cell envelope structure is formed beneath the plasma membrane in terminally differentiating stratified squamous epithelia. It provides a vital physical barrier for invading pathogens and consists of a 10-nm-thick layer of highly cross-linked, highly insoluble structures (42, 43). The envelope is composed of multiple membrane-associated proteins, such as involucrin and cytosolic proteins, including loricrin and the cornin/small proline-rich protein family members. These proteins are cross-linked into an insoluble mesh by keratinocyte TGase, a membrane-bound enzyme. Keratin intermediate filaments at late stages consisting almost entirely of keratin 1, keratin 2e, and keratin 10, become cross-linked to the cornified cell envelope. In contrast to mammalian keratins and keratin-associated proteins, little is known about invertebrate keratins and keratin-associated proteins. A keratin-like protein F46E10.11 from *C. elegans* (29) also exhibits sequence similarity to mammalian high sulfur keratin-associated proteins, especially those with a high Cys content (13.7%) that includes Cys clusters such as Cys-Cys-Arg-Val and Cys-Cys-Pro-Tyr (Fig. 2B).

The epidermis barrier wound repair pathway seems to be evolutionally well conserved between arthropods and mammals. In *Drosophila*, the transcription factor grainy head regu-
lutes production of the enzymes, dopa decarboxylase and tyrosine hydroxylase, which catalyze the production of quinines, leading to covalent cross-linking between cuticular proteins and cuticular structural components (44). Mice lacking a homologue of Drosophila grainy head (Grainy head-like 3) display defective skin barrier function and deficient wound repair accompanied by reduced expression of keratinocyte TGase (45). Our data support an assumption that also in arthropods, TGase-dependent protein cross-linking is involved in the initial stage of wound closure and healing of sub-cuticular epidermis, as in the case of mammalian skin.

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