Proline-rich Cell Surface Antigens of Horseshoe Crab Hemocytes Are Substrates for Protein Cross-linking with a Clotting Protein Coagulin*

Tsukasa Osaki, Nozomu Okino†, Fuminori Tokunaga§, Sadaaki Iwanaga¶, and Shun-ichiro Kawabata||

From the Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan

Monoclonal antibodies were raised against hemocytes of the horseshoe crab Tachypleus tridentatus. All of the antibodies obtained reacted with the same protein bands on SDS-PAGE of hemocyte lysate. Flow cytometry and biotinylation of surface substances on the hemocytes indicated that the antigens are major peripheral proteins of hemocytes. The antigens were purified from hemocyte lysate and were good substrates for the horseshoe crab hemocyte transglutaminase (HcTGase). Transglutaminases play an important role during the final stage of blood coagulation in mammals and crustaceans. Although HcTGase did not intermolecularly cross-link a clottable protein coagulogen or its proteolytic product coagulin, HcTGase promoted the cross-linking of coagulin with the surface antigens, resulting in the formation of a stable polymer. We determined the nucleotide sequences for two isoproteins of the antigens. The two proteins containing 271 and 284 residues (66% identity) were composed of tandem repeats of proline-rich segments. We named them proxins-1 and -2 after proline-rich proteins for protein cross-linking. Proxins may form a stable physical barrier against invading pathogens in cooperation with hemolymph coagulation at injured sites.

Arthropoda lack adaptive immunity, but they are well adapted to diverse environments and effectively defend themselves against invading pathogens by innate immunity. All multicellular organisms have some form of innate immune system. Recent studies (1, 2) have revealed that insects and mammals conserve a signaling pathway of the innate immune system. Recent studies (1, 2) have revealed that insects and mammals conserve a signaling pathway of the innate immune system.

* This work was supported by Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (to S. K.) and a grant-in-aid for Scientific Research of Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank*TM/EBI Data Bank with accession number(s) AB076185 and AB076186.
† Present address: Dept. of Human Genetics, Mount Sinai School of Medicine, New York, NY 10029.
‡ Present address: Dept. of Molecular Cell Biology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan.
¶ Present address: The Chemo-Sero-Therapeutic Research Institute, Kumamoto 860-8568, Japan.
|| To whom correspondence and reprint requests should be addressed: Dept. of Biology, Faculty of Sciences, Kyushu University, Fukuoka, 812-8581, Japan. Tel./Fax: 81-92-642-2633; E-mail: skawaschi@mbox.nc.kyushu-u.ac.jp.

EXPERIMENTAL PROCEDURES

Materials—Coagulogen (8–10) and 8.6-kDa protein (11) were prepared from hemocyte lysates of the horseshoe crab T. tridentatus as described. LPS from Salmonella minnesota R595 was from List Biological Laboratories, Inc. (Campbell, CA).

Preparation of Hemocytes—Hemolymph was collected into 5 volumes of 2% paraformaldehyde in 10 mM sodium phosphate, pH 7.5, containing 0.5 mM NaCl and 0.05% NaN3 (PBS) and allowed to stand for 10 min at 25 °C. The fixed hemocytes were collected by centrifugation at 800 rpm (100 × g) at 4 °C for 5 min and washed three times with PBS. The cell numbers were counted by a Coulter Z1 cell counter (Coulter Electronics, Ltd., Luton, UK).

Preparation of Monoclonal Antibodies—The immunization and hybridoma preparation were carried out at Panapharm Laboratories, Co., Ltd., Kumamoto, Japan. Five mice were immunized by injection of the fixed hemocyte suspension containing 2 × 106 cells emulsified with complete Freund’s adjuvant. Hybridoma cells were plated in microwell plates, and positive hybridomas were screened by enzyme-linked immunosorbent assay (ELISA). Positive hybridomas selected were further analyzed by a FACSScan flow cytometer (BD Biosciences). Briefly, each culture supernatant was incubated with the fixed hemocytes at 106 cells/ml in PBS containing 0.1% bovine serum albumin on ice for 30 min. After washing with the same buffer, the hemocytes were incubated with a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) on ice for 30 min. After washing, the
labeled hemocytes were analyzed using the flow cytometer. For each sample, 1.0 × 10^6 cells were analyzed using Cellquest software.

**Immunofluorescence Microscopy**—The fixed hemocytes were incubated with a monoclonal antibody at 1.0 μg/ml on ice for 30 min. After washing with PBS, the hemocytes were incubated with an Alexa Fluor^TM^ 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) at 20 μg/ml on ice for 30 min. After washing with PBS, fluorescence microscopy was done using an Olympus Fluorescence microscope, model BX-FLA (Olympus Optical Co., Ltd., Tokyo, Japan).

**Biotinylation of Surface Substances of Hemocytes and Immunoprecipitation**—Surface substances exposed on hemocytes were biotinylated using the water-soluble biotinylation reagent, sulfo-N-hydroxysuccinimide long chain biotin (Pierce). Briefly, hemolymph was collected into PBS and the hemolymph plate and adhered to a glass slide. Hemocytes that adhered to the plate were washed three times with PBS and then incubated with the biotinylation reagent (0.5 mg/ml) in PBS at 4 °C for 30 min with rocking. The biotinylation reaction was stopped by incubating with 100 mM glycine in PBS at 4 °C for 20 min. No morphological change of the hemocytes that adhered to the plate was observed microscopically after biotinylation. The hemocytes biotinylated were lysed with 50 mM Tris-HCl, pH 7.5, containing 0.15 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40 at 4 °C for 30 min and the supernatant was obtained by centrifugation. The transglutaminase reaction of the supernatant was started by adding 10 mM CaCl_2 and 10 mM dithiothreitol in the presence or absence of 0.5 mM DCA and incubated at 37 °C. The reaction was terminated by adding 100 mM EDTA. An aliquot of the reaction mixture was subjected to SDS-PAGE, and the fluorescence-labeled proteins were visualized by a trans-illuminator.

**Participation of the HcTGase and Protein Cross-linking of the Hemocyte Surface Antigens**—HcTGase was partially purified as described (11) with some modifications. The freshly prepared hemocyte lysate was dialyzed against 50 mM Tris acetate, pH 7.5, containing 1 mM EDTA. The dialyzed sample was applied to an SP-Sepharose FF column, followed by a DEAE-Sepharose CL-6B column. The transglutaminase activity was detected by DCA incorporation into the 8.6-kDa protein. The partially purified HcTGase (13 μg/ml) was incubated with the hemocyte surface antigens (400 μg/ml) in the presence of 10 mM CaCl_2 and 10 mM dithiothreitol at 37 °C for 1 h. An aliquot of the reaction mixture was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

**Cross-linking of the Hemocyte Surface Antigens with Coagulin**—Coagulogen (2 mg/ml in 50 mM Tris-HCl, pH 7.5, containing 0.15 mM NaCl) was incubated with trypsin (Worthington) at 37 °C for 1 h (enzyme/substrate = 1:250, w/w). The resulting coagulin gel was dissolved by 20-fold dilution with the same buffer to a final concentration of 0.1 mg/ml, and no clot formation was observed under these conditions. The purified coagulin (50 μg/ml) in 50 mM Tris acetate, pH 7.5, containing 10 mM CaCl_2 and 10 mM dithiothreitol, were preincubated with coagulin, coagulin, or 8.6-kDa protein (50 μg/ml) on ice for 20 min. Partially purified transglutaminase (7 μg/ml) was then added to the mixture and incubated at 37 °C for 1 h. An aliquot of the reaction mixture was subjected to electrophoresis using 1% agarose gel, not polyacrylamide gel, under the same conditions of SDS-PAGE, followed by immunoblotting.

**Enzyme-linked Immunosorbent Assay**—Microtiter plates were coated with 10 μg/ml coagulogen or coagulin in 50 mM Tris-HCl, pH 7.5, containing 0.15 mM NaCl, by incubating overnight at 4 °C. After washing with the same buffer, the plates were blocked with 1% bovine serum albumin, and serial dilutions of samples were added, incubated at 37 °C for 2 h, and then washed. A monoclonal antibody was added and incubated at 37 °C for 1 h and washed. Horseradish peroxidase-conjugated goat anti-mouse IgG was added and incubated at 37 °C for 1 h. The enzyme activity of horseradish peroxidase was detected by using polyclonal antibody against HcTGase and the horseradish peroxidase-conjugated goat anti-rabbit IgG. To determine whether significant membrane damage of hemocytes occurred because of LPS, the activity of lactate dehydrogenase, a cytosolic enzyme, was measured by using a colorimetric cytotoxicity assay kit according to the protocol provided by the manufacturer (Oxford Biomedical Research, Inc., Oxford, MI).

**LPS Stimulation of Horseshoe Crab Hemocytes**—Hemolymph, 2 ml, was collected into the sterile tube, and the hemocytes were collected by centrifugation at 800 rpm (100 × g) at 4 °C for 5 min and washed twice with 10 mM HEPES-NaOH, pH 7.0, containing 0.5 mM NaCl. Then the hemocytes were suspended in the same buffer containing 50 mM MgCl_2 and 1% homogenate and serial dilutions were prepared, incubated at 37 °C for 30 min. HcTGase in the supernatant obtained by centrifugation at 800 rpm (100 × g) at 4 °C for 5 min was detected by ELISA, using polyclonal antibody against HcTGase and the horseradish peroxidase-conjugated goat anti-rabbit IgG. To determine whether significant membrane damage of hemocytes occurred because of LPS, the activity of lactate dehydrogenase, a cytosolic enzyme, was measured by using a colorimetric cytotoxicity assay kit according to the protocol provided by the manufacturer (Oxford Biomedical Research, Inc., Oxford, MI).

**RESULTS**

Preparation of Monoclonal Antibodies That Recognize Hemoctye Surface Antigens—Fifteen hybridomas producing monoclonal antibodies reacting with surface substances of the horseshoe crab hemocytes were selected by the two screening methods, ELISA and the flow cytometric analysis. Immunoblotting showed that all the monoclonal antibodies produced by these hybridomas reacted with the protein bands of apparent molecular masses of 90 and 120 kDa in the 1% SDS extract of hemocytes (data not shown). One of the hybridomas cloned by...
limiting dilution, named 6C1-1F, produced an antibody with the highest affinity to the antigens. Flow cytometric analysis confirmed that antibody 6C1-1F reacts with the antigens expressed on the surface of hemocytes (Fig. 1A). The antigens on the hemocytes were visualized by a fluorescence-labeled secondary antibody, Alexa 488-conjugated goat anti-mouse IgG (Fig. 1B).

On the other hand, the cell surface substances of hemocytes were biotinylated and then extracted by 1% SDS and subjected to SDS-PAGE, followed by detection with streptavidin-biotinylated horseradish peroxidase. At least six protein bands were detected with apparent molecular masses ranging from 48 to 170 kDa (Fig. 2, lane 1). The biotinylated cell surface proteins were immunoprecipitated by antibody 6C1-1F and subjected to SDS-PAGE, followed by detection with streptavidin-biotinylated horseradish peroxidase (Fig. 2, lane 2). As a result, the proteins of 90 and 120 kDa were immunoprecipitated, indicating that antibody 6C1-1F recognizes the major hemocyte surface antigens, tentatively named band-90 and band-120.

Purification of Bands-90 and -120—The hemocyte lysate (33.6 g wet weight) was fractionated with solid ammonium sulfate, and bands-90 and -120 were precipitated at the satu-

**Fig. 1. Flow cytometry and immunofluorescence microscopy of hemocytes.** A, the fixed hemocytes were incubated with antibody 6C1-1F (1 µg/ml), followed by fluorescein isothiocyanate-labeled secondary antibody. The dotted line is a negative control without the first antibody. B, the immunofluorescence staining of hemocytes was done as described under “Experimental Procedures.”

**Fig. 2. Surface proteins on hemocytes.** The biotinylation and immunoblotting were done as described under “Experimental Procedures.” Lane 1, biotinylated proteins were visualized with streptavidin-biotinylated horseradish peroxidase complex followed by enhanced chemiluminescence. Lane 2, the surface antigens immunoprecipitated with 6C1-1F antibody were visualized using the same method.

**Fig. 3. Purification of bands-90 and -120.** Experimental details are presented under “Results.” A, Sepharose CL-6B chromatography. B, DEAE-Sephacel chromatography. C, SDS-PAGE of purified bands-90 and -120 under reducing conditions.

| Analysis | Sequence |
|----------|----------|
| Band-90  | Band-120 | Proxin-1 | Proxin-2 |
| Asp      | 11.5     | 11.9     | 11.4 (31) | 9.9 (28) |
| Ser      | 5.1      | 4.9      | 4.8 (13)  | 4.9 (14)  |
| Glu      | 13.6     | 13.5     | 17.3 (47) | 13.0 (37) |
| Gly      | 6.9      | 6.8      | 4.4 (12)  | 4.9 (14)  |
| His      | 6.5      | 6.3      | 5.2 (14)  | 5.6 (16)  |
| Arg      | 6.4      | 6.4      | 5.5 (15)  | 5.3 (15)  |
| Thr      | 1.7      | 1.5      | 4.1 (11)  | 4.2 (12)  |
| Ala      | 0.2      | 0.3      | 0.0 (0)   | 0.7 (2)   |
| Pro      | 17.4     | 18.2     | 17.7 (48) | 19.7 (56) |
| 1/2Cys  | ND       | ND       | 0.7 (2)   | 0.7 (2)   |
| Tyr      | 2.4      | 2.3      | 3.0 (8)   | 2.8 (8)   |
| Val      | 6.8      | 6.5      | 7.0 (19)  | 7.4 (21)  |
| Met      | 0.0      | 0.0      | 0.0 (0)   | 0.4 (1)   |
| Lys      | 6.7      | 7.0      | 6.6 (18)  | 6.3 (18)  |
| Ile      | 5.7      | 5.7      | 3.7 (10)  | 6.3 (18)  |
| Leu      | 8.8      | 8.4      | 8.5 (23)  | 7.8 (22)  |
| Phe      | 0.1      | 0.2      | 0.0 (0)   | 0.0 (0)   |
| GlcNH₂   | –        | –        | –         | –         |
| GalNH₂   | +        | +        | –         | –         |

**Table I**

Amino acid compositions of band-90 and band-120

The values in parentheses are the residue numbers deduced from the nucleotide sequences. ND indicates not determined; – indicates not detectable; and + indicates detectable.
of Bands-90 and -120 could not be separated by these purification procedures (Fig. 3A). DCA incorporation was performed in the presence of 100 mM CaCl$_2$ at 37 °C for 60 min (lanes 1–6). DCA incorporation was catalyzed by an intrinsic HcTGase that was dialyzed against 50 mM Tris acetate, pH 7.5, containing 0.03 M NaCl and 1 mM EDTA and applied to DEAE-Sephacel column (2.5 × 10 cm) equilibrated with the same buffer. After washing with the equilibration buffer, the proteins were eluted with a linear gradient of 0.03–0.6 M NaCl in the same buffer (Fig. 3B). Immunoblot analysis indicated that bands-90 and -120 were eluted in the flow-through fraction. Bands-90 and -120 could not be separated by these purification procedures (Fig. 3C).

**Amino Acid Sequence and Amino Acid Composition Analyses of Bands-90 and -120**—The NH$_2$-terminal sequence analysis of bands-90 and -120 transferred to a membrane proved to be identical at least up to 23 residues. The transferred proteins were digested with Asp-N protease or chymotrypsin, and their peptide mappings were done by rpHPLC. As far as they were examined, the peptide sequences derived from band-90 were identical to those from band-120, and the amino acid compositions of bands-90 and -120 were indistinguishable (Table I). Band-120 is possibly a post-translationally modified protein of band-90. Bands-90 and -120 were subjected to SDS-PAGE after incubation at 37 °C for 0, 5, 10, 20, 30, and 60 min (lanes 1–6). DCA incorporation was performed in the presence of 100 mM CaCl$_2$ at 37 °C for 60 min (lane 7). After DCA labeling, aliquots of the reaction mixture were immunoprecipitated with antibody 6C1-1F, followed by SDS-PAGE. Proteins labeled with DCA on gels were detected by UV illumination.

**DCA Incorporation into Bands-90 and -120**—The NH$_2$-terminal sequence similarity with that of the 80-kDa protein previously identified in *T. tridentatus* hemocytes (11). The partial NH$_2$-terminal sequence reported for the 80-kDa protein (SVXTLQV) was identical to that of band-90 or band-120 (HVKTLQV), except for the NH$_2$-terminus. The 80-kDa protein is a substrate for HcTGase that catalyzes the DCA incorporation into the 80-kDa protein in a Ca$^{2+}$-dependent manner. To test whether DCA is incorporated into the surface antigens, the hemocytes lysate was incubated with DCA in the presence of CaCl$_2$, and aliquots were subjected to SDS-PAGE at the indicated times. DCA incorporation into the several specific proteins was observed by UV illumination of the gel, and the proteins of 90- and 120-kDa were most reactive among these DCA-labeled ones (Fig. 4A). The incorporation reaction proceeded rapidly (<5 min), and it was completely inhibited by EDTA, indicating that the intrinsic HcTGase catalyzes the DCA incorporation. Furthermore, the 90- and 120-kDa proteins labeled with DCA were immunoprecipitated by antibody 6C1-1F (Fig. 4B), indicating that bands-90 and -120 are substrates for HcTGase and function as amine acceptors.

**Cross-linking of Bands-90 and -120 with Coagulin**—To test whether bands-90 and -120 are cross-linked with endogenous proteins, the freshly prepared hemocyte lysate was incubated in the presence of CaCl$_2$, and aliquots were immunoprecipitated by antibody 6C1-1F and then subjected to immunoblotting. Two protein bands with apparent molecular masses of 140 and 200 kDa newly appeared after a 5-min incubation. These bands were not observed in the presence of EDTA (data not shown). Furthermore, the incubation of the purified bands-90 and -120 with HcTGase resulted in the cross-linked products of 140 and 200 kDa (data not shown). These results indicate that bands-90 and -120 are cross-linked intermolecularly by HcTGase.

The final stage of the blood coagulation cascade in mammals (17) and the hemolymph coagulation reactions in crustaceans (18) depends on the transglutaminase-mediated cross-linking of specific clotting proteins. However, HcTGase does not cross-link a clottable protein coagulin of the horseshoe crab (11). Coagulogen is converted to an insoluble gel known as coagulin polymer through the proteolytic cascade triggered by LPS. HcTGase neither catalyzes DCA incorporation into coagulin nor cross-linked it intermolecularly (data not shown). Interestingly, HcTGase promoted cross-linking of coagulin with bands-90 and -120, resulting in the high molecular weight products located at the top of the gel by 1% agarose gel electrophoresis in the presence of SDS (data not shown). Coagulogen or 8.6-kDa protein, a previously identified substrate for HcTGase (11), did not result in the high molecular weight products cross-linked with bands-90 and -120 by HcTGase. In the absence of HcTGase bands-90 and -120 non-covalently bound to coagulin but not coagulogen-coated on microtiter plates, suggesting that bands-90 and -120 have specific binding affinity to coagulin (Fig. 5). To determine whether HcTGase could be released from hemocytes in response to external stimuli, hemocytes were
treated with LPS. HcTGase was released from hemocytes into the extracellular fluid in response to the stimulation by LPS, as detected by ELISA (data not shown). The lactate dehydrogenase activity in the extracellular fluid was not detectable under the same conditions.

**Nucleotide Sequences of Bands-90 and -120**

The specific probe of 0.45 kb was identified with oligonucleotide primers corresponding to peptides derived from band-90 or band-120, using PCR and DNA sequence analyses. Screening a hemocyte cDNA library with the probe gave two types of positive clones that code the full length of the protein sequences. They were subjected to restriction mappings followed by sequence determinations of both strands. One cDNA included an open reading frame of 874 nucleotides with a mature protein of 271 residues, and the other included an open reading frame of 926 nucleotides with a mature protein of 284 residues; the overall sequence was determined.

**Fig. 6. Nucleotide sequences of proxins-1 and -2.** A, a nucleotide sequence of proxin-1. B, a nucleotide sequence of proxin-2. Single underlines represent the amino acid sequences determined by peptide sequencing, and asterisks represent the stop signal.
The intriguing feature of these sequences was the presence of four tandem repeats with an extremely high content of proline, accounting for 18 and 20% of the total residues, respectively (Fig. 7). Therefore, we named them proxin-1 and proxin-2, respectively, after proline-rich proteins for protein cross-linking. There were glutamine-rich regions containing Gln-Gln dipeptides at the NH2-terminal regions of proxins-1 and -2 and the COOH-terminal region of proxin-1. The calculated molecular weights from the deduced sequences of proxins-1 and -2 were 30,899 and 31,890, respectively. Peptide fragments corresponding to only proxin-1 or proxin-2 were obtained from the proteolytic digest of band-90 and from that of band-120 by rpHPLC, indicating that not only band-90 but also band-120 contains both proxins-1 and -2. Several peptide fragments were isolated with low yields, and their sequences were homologous but not identical to those of proxins-1 and -2, indicating the presence of an unidentified isoprotein(s) for the proxins. The proxins did not have putative transmembrane domains or glycosylphosphatidylinositol anchor sites in their sequences (19).

**Tissue-specific Localization of Proxins**—Immunoblotting with antibody 6C1-1F detected the proxins only in hemocytes but not in other tissues, including heart, skeletal muscle, hepatopancreas, and stomach (Fig. 8A). Furthermore, reverse transcription PCR showed that the cDNA fragments corresponding to proxins were highly amplified only in hemocytes but not in other tissues, thus indicating the specific expression of proxins in hemocytes (Fig. 8B).

**DISCUSSION**

In mammals, the coagulation system is based on the proteolytically induced aggregation of fibrinogen into insoluble fibrin (20–22). Initially, fibrins are non-covalently associated and are further stabilized through the intermolecular cross-linking of ⩾Glu-lysine bonds by the plasma transglutaminase. On the other hand, in crustaceans, hemolymph coagulation depends on the transglutaminase-mediated cross-linking of a specific plasma-clotting protein without the proteolytic cascade (18, 23). In the horseshoe crab, the coagulation cascade, whose components have structural similarity to those of the morphogenetic cascade for determining embryonic dorsal-ventral polarity in *Drosophila*, is activated by LPS or β-1,3-glucans, leading to the conversion of a soluble coagulogen into an insoluble coagulin gel (5, 6). No transglutaminase activity has been found in horseshoe crab plasma. However, the clots of whole blood yielded significant amounts of ⩾Glu-lysine products, suggesting the activity of transglutaminase released from the hemocytes during coagulation (24). We identified here the proline-rich surface antigens of the horseshoe crab hemocytes, named proxins, that function as substrates for protein cross-linking with clotting protein coagulin. These results clearly indicate that the horseshoe crab also uses the protein cross-linking reaction at the final stage of the coagulation system as an important host defense system.

The apparent molecular masses of proxins on SDS-PAGE are definitively higher than those deduced from the cDNA sequences. Proxins-1 and -2 contained small amounts of galactosamine and no glucosamine in hydrolysates of bands-90 and -120 by amino acid analysis (Table I). Therefore, the glycosylation may not explain the big difference between the molecular weights of proxins estimated by the sequence and those estimated by SDS-PAGE. Bands-90 and -120 may consist of cross-linked oligomers of proxins-1 and -2.
In mammals, proline-rich proteins such as cornifins and small proline-rich proteins are involved in the formation of the cornified cell envelope, a highly insoluble structure at the cell periphery of the stratum corneum (25, 26). The stratum corneum of the skin serves as a forefront physical barrier for invading pathogens. The envelope is composed of multiple membrane-associated and cytosolic proteins, including members of the cornifin/small proline-rich protein family and various other proteins. These proteins are cross-linked into an insoluble mesh by the keratinocyte transglutaminase, a membrane-bound enzyme. Cornifins function as terminal glutamine-rich portion and proline-containing tail, a casting net, and an important physical barrier for invading microbes.

Proxins may participate in forming the stable reticulate structure, a casting net, and an important physical barrier for invading microbes.

Acknowledgments—We thank W. Kamada, M. Hirang-Kawabata, and N. Ichinomiya for expert technical assistance with peptide sequencing and amino acid analysis.

REFERENCES
1. Lemaître, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996) Cell 86, 973–983
2. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997) Nature 388, 394–397
3. Söderhall, K., and Cerenius, L. (1998) Curr. Opin. Immunol. 10, 23–28
4. Ashida, M., and Brey, P. T. (1998) in Molecular Mechanisms of Immune Responses in Insects (Brey, P. T., and Hulmanson, D., eds) pp. 135–172, Chapman and Hall Ltd., London
5. Iwanaga, S., Kawabata, S., and Muta, T. (1998) J. Biochem. (Tokyo) 123, 1–15
6. Iwanaga, S. (2002) Curr. Opin. Immunol. 14, 87–95
7. Tosh, Y., Mizutani, A., Tokunaga, F., Muta, T., and Iwanaga, S. (1991) Cell Tissue Res. 266, 137–147
8. Nakamura, S., Iwanaga, S., Harada, T., and Niwa, M. (1976) J. Biochem. (Tokyo) 80, 1011–1021
9. Nakamura, S., Takagi, T., Iwanaga, S., Niwa, M., and Takahashi, K. (1976) Biochem. Biophys. Res. Commun. 72, 902–908
10. Takagi, T., Hokama, Y., Miyata, T., Morita, T., and Iwanaga, S. (1984) J. Biochem. (Tokyo) 95, 1445–1457
11. Tokunaga, F., Yamada, M., Miyata, T., Ding, Y. L., Hirang-Kawabata, M., Muta, T., Iwanaga, S., Ichinose, A., and Davie, E. W. (1993) J. Biol. Chem. 268, 252–261
12. LeGendre, N., and Matsudaira, P. T. (1989) in A Practical Guide to Protein and Peptide Purification for Microsequencing (Matsudaira, P. T., ed) pp. 49–49, Academic Press Inc., San Diego
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Okino, N., Kawabata, S., Saioto, T., Hirata, M., Takagi, T., and Iwanaga, S. (1995) J. Biol. Chem. 270, 31008–31015
15. Laemmli, U. K. (1970) Nature 227, 680–685
16. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
17. Lorand, L., Credo, R. B., and Janus, T. J. (1981) Methods Enzymol. 80, 323–341
18. Kopacek, P., Hall, M., and Söderhall, K. (1993) Eur. J. Biochem. 213, 591–597
19. Cross, G. A. (1990) Annu. Rev. Cell Biol. 6, 1–39
20. Dodtittle, R. F. (1984) in The Plasma Proteins (Putnam, F. W., ed) 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Furie, B., and Furie, B. C. (1988) Cell 53, 505–518
22. Dairie, E. W., Fujikawa, K., and Kisiel, W. (1991) Biochemistry 30, 10363–10370
23. Dodtittle, R. F., and Riley, M. (1990) Biochem. Biophys. Res. Commun. 167, 16–19
24. Wilson, J., Rickles, F. R., Armstrong, P. B., and Lorand, L. (1992) Biochem. Biophys. Res. Commun. 188, 655–661
25. Steiner, P. M., and Marekow, L. N. (1995) J. Biol. Chem. 270, 17792–17711
26. Robinson, N. A., Lapic, S., Welte, J. F., and Eckert, R. L. (1997) J. Biol. Chem. 272, 12035–12046
27. Austin, S. J., Fujimoto, W., Marvin, W. K., Vollberg, T. M., Lorand, L., and Jetten, A. M. (1996) J. Biol. Chem. 271, 3737–3742
28. Kawasaki, H., Nose, T., Muta, T., Iwanaga, S., Shimohigashi, Y., and Kawabata, S. (2000) J. Biol. Chem. 275, 35287–35301
29. Bergner, A., Oganesyan, V., Muta, T., Iwanaga, S., Typke, D., Huber, R., and Bode, W. (1996) EMBO J. 15, 6789–6797