The prophenoloxidase (proPO) cascade is a major innate immune response in invertebrates, which is triggered into its active form by elicitors, such as lipopolysaccharide, peptidoglycan, and 1,3-β-D-glucan. A key question of the proPO system is how pattern recognition proteins recognize pathogenic microbes and subsequently activate the system. To investigate the biological function of 1,3-β-D-glucan pattern recognition protein in the proPO cascade system, we isolated eight different 1,3-β-D-glucan-binding proteins from the hemolymph of large beetle (Holotrichia diomphalia) larvae by using 1,3-β-D-glucan immobilized column. Among them, a 20- and 17-kDa protein (referred to as Hd-PGRP-1 and Hd-PGRP-2) show high sequence identity with the short forms of peptidoglycan recognition proteins (PGRPs-S) from human and Drosophila melanogaster. To be able to characterize the biochemical properties of these two proteins, we expressed them in Drosophila S2 cells. Hd-PGRP-1 and Hd-PGRP-2 were found to specifically bind both 1,3-β-D-glucan and peptidoglycan. By BIACore analysis, the minimal 1,3-β-D-glucan structure required for binding to Hd-PGRP-1 was found to be laminaritetraose. Hd-PGRP-1 increased serine protease activity upon binding to 1,3-β-D-glucan and subsequently induced the phenoloxidase activity in the presence of both 1,3-β-D-glucan and Ca2+\(^{2}\), but no phenoloxidase activity was elicited under the same conditions in the presence of peptidoglycan and Ca2+\(^{2}\). These results demonstrate that Hd-PGRP-1 can serve as a receptor for 1,3-β-D-glucan in the insect proPO activation system.

The innate immune system is a host defense mechanism that is evolutionarily conserved from plants to humans and is mainly involved in the recognition and control of the early stage of infection in all animals (1, 2). It is activated by a group of germ line encoded receptors, conceptually termed pattern recognition receptors, that recognize microbial surface determinants that are conserved among microbes but absent in the host, such as lipopolysaccharide (LPS),\(^{3}\) peptidoglycan (PGN), 1,3-β-D-glucan, and mannan. Upon recognition, these receptors activate distinct signaling cascades that regulate specific immune-related proteins aimed at the aggressors. Recently, our knowledge of innate immunity in mammalian and insects has increased dramatically (1–5). The recruitment of similar receptors and pathways in both insects and mammals in the fight against infection suggests that they have developed similar mechanisms and molecular pathways to recognize and eliminate invaders (1, 6).

Peptidoglycan recognition proteins (PGRPs) have been recognized as an important component of the innate immune system in a variety of organisms ranging from invertebrates to vertebrates including insects and mammals. Ashida and colleagues (7) were first to purify a soluble PGRP showing an affinity for PGN, and upon binding to PGN this complex induced activation of the prophenoloxidase (proPO) cascade. Subsequent identification and cloning of many other PGRPs demonstrated that PGRPs are conserved from insects to mammals (8–10). However, the exact biological functions of short forms of PGRPs were not determined.

The proPO activation pathway, like the vertebrate complement system, is a proteolytic cascade comprising pattern recognition proteins, several serine proteases, their inhibitors, and terminates with the zymogen, proPO (11–13). It is known that microbial carbohydrates such as LPS, PGN, or 1,3-β-D-glucan are first recognized by pattern recognition proteins, which then induce activation of serine proteases within the proPO system (11–13). The proPO-activating enzyme or factor, which all are similar to Drosophila easter-type serine proteases, cleaves proPO to generate the active enzyme, phenoloxidase (PO) (14–17). This enzyme produces toxic compounds to microorganisms by oxidizing phenols to melanin, and it also participates in the sclerotization of the cuticle, which is vital for the survival of insects (18). Many reports have been published about the proPO of the invertebrate and its activation mechanism (11–24). A key question regarding the proPO activation system is how pattern recognition molecules can induce activation of the system in response to microbial infection.

We were able to reconstitute the downstream components of the proPO cascade using biochemically purified proteins from a

\(^{3}\) The abbreviations used are: LPS, lipopolysaccharide; PGN, peptidoglycan; PGRP, peptidoglycan recognition protein; proPO, prophenoloxidase; PO, phenoloxidase; ESGC, the eluate solution from 1,3-β-D-glucan-immobilized column; GRP, 1,3-β-D-glucan recognition protein; HPLC, high performance liquid chromatography; bovine OBP, bovine oligosaccharide-binding protein; Boc-Phe-Ser-Arg-MCA, \(\text{N}-\text{butyloxycarbonyl-benzy}-\text{l-L-phenylalanyl-L-seryl-L-arginine 4-methylcoumaryl-7-amide.}\)
large beetle, *Holotrichia diomphalia* (19). Using this in vitro system, we were able to show that the downstream part of the proPO system is regulated by two easter-type serine proteases and a masquerade-type serine protease homologue and involves a two-step proteolysis of proPOs before they exhibit any PO activity. However, the molecular mechanism of the upstream part of the proPO cascade is still poorly understood. Additionally, it remains to be elucidated how the connection between the upstream and downstream part of the proPO cascade is organized and activated. It was proposed that pattern recognition molecules, such as PGRPs, Gram-negative bacteria-binding proteins, LPS, or 1,3-β-d-glucan-binding proteins (GRPs), make a complex with the proPO-activating enzyme(s) and microbial cell wall components, then activate proPO-activating enzyme(s), which then will convert proPO to active PO by a limited proteolysis (11–13, 19, 20, 24).

Recently, we demonstrated that a mixture of plasma and hemocyte lysate of the coleopteran insect *Tenebrio molitor* could induce proPO activation in response to 1,3-β-d-glucan, but not to LPS and PGN (24). This result suggests that this mixture contains all the necessary components for 1,3-β-d-glucan-dependent proPO activation, such as 1,3-β-d-glucan pattern recognition protein(s), proPO-activating factors (PAAFs), and proPOs. In this article we report that *Drosophila* PGRP-SA-like proteins from the large beetle *H. diomphalia* recognize 1,3-β-d-glucan and induce 1,3-β-d-glucan-dependent proPO activation with the increase of serine protease activity upon binding to 1,3-β-d-glucan in the presence of Ca²⁺. These proteins were also expressed in *Drosophila* S2 cells, and their biochemical properties were studied.

### EXPERIMENTAL PROCEDURES

**Animals and Collection of Hemolymph—**Methods for rearing the insects and collection of hemolymph were as previously described (25). Hemocytes were collected from the hemolymph by centrifugation at 200 × g for 10 min at 4 °C, washed with anti-coagulation buffer (30 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 4.6, buffer A), and stored at −80 °C. Approximately 3 × 10⁹ packed cells were obtained from 500 ml of hemolymph. The supernatant was taken as plasma, adjusted to pH 4.6 with 1 M citric acid solution, and stored at −80 °C until use. The hemocyte lysate was prepared according to our previously published method (25). Briefly, 5 ml of packed cells were suspended in 10 ml of 50 mM Tris-HCl, 1 mM EDTA, pH 6.5, and then subjected to ultrasonification five times for 3 s at 4 °C. The suspended solution was centrifuged at 22,900 × g for 20 min at 4 °C. The supernatant was used as hemocyte lysate in further experiments.

**Preparation of 1,3-β-D-Glucan- and PGN-immobilized Columns—**A 1,3-β-d-glucan immobilized column was prepared with a previously published method (26). Briefly, three ml of insoluble 1,3-β-d-glucan (curdian, Wako) stock solution (500 mg of curdian was dissolved in 10 ml of 1 N NaOH solution) was added to 20 ml of 0.2 M KH₂PO₄, and then adjusted to pH 8.4 with 6 M HCl. Two and a half grams of suction-dried AF-Amino Toyopearl 650M was suspended in the soluble curdian solution prepared with 1 N NaOH. After addition of 1 g of NaCNBH₃, the suspension was incubated at 60 °C overnight. The gel was then acetylated to block remaining free amino groups by incubation with 8 ml of 0.2 N sodium acetate and 4 ml of acetic anhydride on ice for 30 min, followed by adding an additional 4 ml of acetic anhydride, and then incubating 30 min at room temperature. Finally, the resin was washed with 150 ml of 0.1 N NaOH, 150 ml of 1 N Tris-HCl, pH 8.0, and finally with distilled water. As a control, the resin was treated as described above but without curdian. The amount of coupled 1,3-β-d-glucan to the resin was quantified by the sulfuric acid-phenol method (27). The resins that had coupled more than 3 μg of glucose/mg of resin were used for further purification of *Holotrichia* 1,3-β-glucan-binding protein. We have made a PGN-immobilized resin by coupling soluble PGN fragments to CNBr-activated Sepharose. The soluble PGN fragments were prepared from *Staphylococcus aureus*-insoluble PGN (Fluka, catalog no. 77140) according to the published method (28).

**Preparation of G-100 Solution and Glucan-specific Solution—**G-100 solution from *Holotrichia* plasma was prepared by our previously published method (29). Briefly, the collected plasma solution (~100 ml containing 900 mg of protein) was concentrated by ultrafiltration using Amicon, YM1. Approximately 30 ml of the concentrated solution was applied to a Sephadex G-100 column (2.0 × 42 cm) equilibrated with buffer B (50 mM Tris-HCl, containing 20 mM EDTA, pH 6.5) and then eluted with the same buffer at a flow rate of 12 ml/h. Fractions (~12 ml containing 100 mg of protein) showing PO activity in the presence of 5 mM CaCl₂ were pooled and concentrated to 5 ml by ultrafiltration. This solution (referred as G-100 solution) was stored at −70 °C until use. To examine the β-1,3-glucan-dependent PO activity, we prepared a solution (referred to as glucan-specific solution) by mixing G-100 solution (4 g of protein) from plasma and the crude hemocyte lysate (4 g of protein).

**Assay of PO Activity—**An assay of PO was carried out according to our previously published method and activity assay (14). Briefly, to measure PO activity, 30 μl of the glucan-specific solution (300 μg of protein) or fractionated G-100 solution (150 μg of protein) or hemocyte lysate (150 μg of protein) was pre-incubated in 70 μl of 20 mM Tris-HCl buffer, pH 8.0, containing 1 mg of 1,3-β-glucan for 10 min at 30 °C, and then 400 μl of substrate solution (1 mM 4-methylcatechol, 2 mM 4-hydroxyproline ethylester in 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM CaCl₂) was added to the reaction mixture. After incubation at 30 °C for 10 min, the increase in absorbance at 520 nm was measured using a Shimadzu spectrophotometer. One unit of phenoloxidase activity was defined as the amount of enzyme causing an increase in absorbance of 0.1 at 520 nm per 10 min of incubation (A₅₂₀/10 min). Laminarinoligosaccharides for examining the glucan-specific PO activity were purchased from Sekigaku Corp. (Tokyo, Japan).

To examine the effects of the eluate solution from 1,3-β-d-glucan-immobilized column (referred to as ESGC) on PO activity, 1,3-β-d-glucan-binding proteins depleted solution was obtained by passing the glucan-specific solution through the 1,3-β-d-glucan-immobilized column. To determine the effects of the purified recombinant Hd-PGRP-1 and Hd-PGRP-2 on the *Holotrichia* proPO system, the purified Hd-PGRPs (1 μg) was incubated with the glucan-specific solution (150 μg of protein), or the eluate solution or the pass-through solution (150 μg of protein) from the 1,3-β-d-glucan-immobilized column at 30 °C for 20 min in the presence of Ca²⁺ and 1,3-β-d-glucan as described above.

**Measurement of the Amidase Activity during ProPO Activation—**To determine amidase activity in the samples, commercially available tryptic substrate (β-butyloxy carbonyl benzyl-l-phenylalanyl-l-seryl-l-arginine 4-methylcoumarylamide-7-amide) (Boc-Phe-Ser-Arg-MCA) was used. In our previous studies, we observed that this substrate was mostly hydrolyzed during *Holotrichia* proPO activation in the presence of 1,3-β-d-glucan and calcium ion (14, 30). This substrate was dissolved in dimethylformamide according to the instruction from the manufacturer. One hundred μl of reaction mixture for measuring PO activity was incubated with 490 μl of substrate solution, which contains 40 μM substrate in 20 mM Tris-HCl buffer, pH 8.0. After incubation of the mixture at 30 °C for 1 h, 500 μl of 17% (v/v) acetic acid was added to terminate enzymatic activity. The activity of the amidase was detected by a fluorescence spectrophotometer at λₑₓ = 380 nm and λₑₘₐₓ = 460 nm. As a control, 100 μl of buffer A was added to check amidase activity as above. One unit of the amidase activity was defined as the amount that liberated 1 nmol of 7-amino-4-methylcoumarin/min.

**Purification of the Denatured Holotrichia 1,3-β-D-Glucan-binding Proteins—**To purify proteins that can recognize 1,3-β-glucan from the hemolymph of *H. diomphalia* larvae, the glucan-specific solution was applied to a 1,3-β-d-glucan-immobilized Toyopearl column (2.5 × 5 cm) equilibrated with buffer B at 0.6 ml/min. After the washing column with buffer B until no absorbance at 280 nm was evident, bound proteins were eluted with buffer B containing 8 M urea at 0.5 ml/min. Then they were further purified by SDS-PAGE under reducing conditions. The enriched two bands with molecular sizes of 20 and 17 kDa were cut out from the gel and then extracted by electroelution with 250 mA for 3 h at 4 °C according to the instructions from the manufacturer. Finally the purity of the two bands was checked by SDS-PAGE under reducing conditions.

**To determine the partial amino acid sequences of the purified 20- and 17-kDa proteins, the purified proteins (25 μg each) were reduced, alkylated, and digested with 2 μg of lysylendopeptidase at 37 °C for 13 h. The digested peptides were separated by HPLC on a C₄r reverse phase column (Gilson) with a linear gradient between 0.05% trifluoroacetic acid in water and 0.052% trifluoroacetic acid in 80% acetonitrile (30). The resulting amino-terminal amino acid sequence of the digested peptides was determined by an Applied Biosystem Procise automated gas-phase amino acid sequencer (31).

**cDNA Cloning and Nucleotide Sequencing of the 20- and 17-kDa Proteins—**A cDNA library from *H. diomphalia* larvae was constructed...
as previously described (14) by using a ZAP-cDNA synthesis kit (Stratagene). Three partial amino acid sequences of the purified 20-kDa protein were determined. Among them, an oligonucleotide corresponding to IQNWEDPT was synthesized as follows: 5′-ATTCGAA/CA/CAAGGAT/TC/AC-3′, and it was labeled with [γ-32P]-ATP using a previously described method (32). Additionally, three partial amino acid sequences of the purified 17-kDa protein were determined. Among them, an oligonucleotide corresponding to NBWGGQQQA was synthesized as follows: 5′-AA/TG/GGTTCGCA/A/G/GCA/GGC-3′, and it was also labeled with [γ-32P]-ATP. We screened $5 \times 10^3$ colonies and obtained six hybridization-positive clones. We analyzed two plasmids containing two chemically determined amino acid sequences and amino-terminal sequences. The deduced amino acid sequences of the 20-kDa (Hd-PGRP-1) and 17-kDa (Hd-PGRP-2) proteins were compared with the protein sequence data base of the National Center for Biotechnology Information using the Genetyx system (Software Development Co., Ltd., Tokyo, Japan).

Expression and Purification of Recombinant Proteins in Drosophila
Schneider S2 Cell Line—The open reading frames corresponding to the full length of Hd-PGRP-1 and Hd-PGRP-2 proteins were subcloned into pMT/Bip/V5-His vector (pMT/Bip/V5-His-Hd-PGRPs-1 and -Hd-PGRP-2) under control of the metallothionen promoter (Invitrogen). Stable cell lines expressing Hd-PGRP-1 and Hd-PGRP-2 proteins were generated by transfection of each pMT/Bip/V5-His clone into Drosophila Schneider S2 cells. To detect secretion of recombinant proteins, we used a goat anti-V5 antibody at the COOH-terminal tag. The secreted recombinant fusion proteins were examined with the anti-V5 antibody (Invitrogen) by Western blot analysis. Then the recombinant Hd-PGRP-1 and Hd-PGRP-2 proteins secreted into the medium were purified with MagNeHis32 nickel particles according to the instructions from the manufacturer (Promega).

RESULTS

Relationship between PO Activity and Amidase Activity of 1,3-β-D-Glucan-dependent ProPO Activation—It is well known that invertebrate proPO system can be activated by LPS, PGN, and 1,3-β-D-glucan (11–13). To purify 1,3-β-D-glucan recognition protein(s) from Holotrichia proPO system, it is necessary to make a solution showing PO activity by 1,3-β-D-glucan, not by PGN or LPS. As shown in Fig. 1A, we prepared a glucan-specific solution showing 1,3-β-D-glucan-dependent PO activities using a mixture of G-100 solution from plasma and hemocyte lysate. This solution specifically showed PO activity in the presence of both 1,3-β-D-glucan and Ca2⁺ (column 10), but not in the presence of Ca2⁺ and PGN or LPS (columns 11 and 12). This result suggests that the glucan-specific solution contains all necessary proPO-activating enzymes, proPOs, and unidentified 1,3-β-D-glucan recognition protein(s).

Previously it was reported that insect proPO system induced the activation of serine protease zymogen to active serine protease during 1,3-β-D-glucan-dependent proPO activation (11–24, 30). To explore the existence of serine protease activity during 1,3-β-D-glucan-specific proPO activation, we examined the presence of serine protease activity bound to the 1,3-β-D-glucan (column 11) and Ca2⁺ (column 12) using commercially available trypsin substrate, Boc-Phe-Arg-MCA. As shown in Fig. 1B, the hemocyte lysate did not show any amidase activities even in the presence of Ca2⁺ and 1,3-β-D-glucan (column 8). When Ca2⁺ ion was added to G-100 solution, amidase activity increased compared with the G-100 alone (column 3). By addition of hemocyte lysate to G-100 solution in the presence of Ca2⁺, more amidase activity was increased (column 6). However, when 1,3-β-D-glucan was added to the mixture of hemocyte lysate and G-100 solution in the presence of Ca2⁺, the amidase increased 2-fold compared with that of Ca2⁺ only (column 10). These results suggest that 1,3-β-D-glucan might increase the amidase activity by activation of the unidentified serine protease zymogen to active serine protease or that some hemocyte lysate protein(s) recognizing 1,3-β-D-glucan will enhance the activation of zymogen serine protease. Interestingly, even though the endogenous amidase activities were observed in the presence of PGN or LPS (columns 11 and 12), PGN- or LPS-dependent PO activity was not observed, suggesting that PGN or LPS pattern recognition protein(s) or cofactor(s) will be necessary to show PGN- or LPS-specific PO activity. These factors might be excluded during the preparation of G-100 solution or hemocyte lysate from the crude hemolymph.

Eight 1,3-β-D-Glucan-binding Proteins Were Purified from the Glucan-specific Solution—To purify the 1,3-β-D-glucan recognition protein(s) of the glucan-specific solution, we have loaded the glucan-specific solution to a 1,3-β-D-glucan-coupled Toyopearl column. The pass-through solution or ESGC of 1,3-β-D-glucan column did not show any PO activity in the presence of 1,3-β-D-glucan and Ca2⁺ (data not shown). This result suggests that Holotrichia 1,3-β-D-glucan recognition protein(s) was absorbed to the 1,3-β-D-glucan-coupled column. When the column was eluted with $3 \times 10^3$ NaCl, small amounts of proteins were eluted from the column (lane 3 in Fig. 2A) and with buffer B

affinity-purified antibodies against Hd-PGRP-1 and Hd-PGRP-2 (50 μg/ml) and then incubated at 4°C for 2 h. The bound antibody was identified using an ECL Western blotting reagent kit.

Determination of Hd-PGRP Localization—Hemolymph and fat body were obtained from 5 larvae. The hemolymph was centrifuged separately at 3,000 rpm at 4°C for 10 min. The supernatant was used as plasma. The hemocyte lysate was prepared as described above. The soluble proteins of plasma, hemocyte lysate, and fat body were precipitated with trichloroacetic acid and subjected to SDS-PAGE and then immunoblotting with affinity-purified Hd-PGRP-1 and Hd-PGRP-2 antibodies, respectively.

1,3-β-D-Glucan- or PGN-immobilized resins in 50 mM Tris-HCl, pH 7.0, at 4°C overnight with rocking. After centrifugation, the resins were washed three times with the same buffer. Proteins bound to the 1,3-β-D-glucan (column 6) and Ca2⁺ (column 12) were eluted from the column 6 and 12, respectively. These results suggest that 1,3-β-D-glucan-specific solution contains all necessary proPO-activating enzymes, proPOs, and unidentified 1,3-β-D-glucan recognition protein(s).
containing 8 M urea specifically glucan-binding proteins could be eluted. Eight proteins in the 8 M urea elution solution were enriched compared with the crude plasma and hemocyte lysate solution (lane 4 in Fig. 2A). When the 8 M urea elution solution was analyzed by SDS-PAGE under non-reducing conditions, bands 7 and 8 were found to be mostly enriched (lane 4 in Fig. 2A and B).

To characterize the biochemical properties of these proteins, the eight eluted proteins were blotted to polyvinylidene difluoride membrane and their amino-terminal sequences or partial amino acid sequences were determined. The amino-terminal sequences of bands 1, 2, 4, and 6 were blocked. The partial amino acid sequences of bands 2 and 4 did not show any homology with known proteins. However, the partial amino acid sequence of band 5 showed an identity with that of Holotrichia proPO-activating factor II that was previously shown to be involved in Holotrichia proPO activation (Ref. 25, data not shown). Interestingly, the amino-terminal sequences of bands 7 and 8 showed high identities with those of Drosophila peptidoglycan recognition protein-SA (Fig. 2C). Bands 7 and 8 had molecular masses of 20 and 17 kDa under reducing conditions, respectively (lane 4 in Fig. 2A), whereas under non-reducing conditions the mass was ~16 kDa for both (lane 4 in Fig. 2B). We have named bands 7 and 8 as H. diomphalia peptidoglycan

Fig. 1. PO activity (A) and amidase activity (B) of G-100 solution and hemocyte lysate. PO activity and amidase activity were measured as described under “Experimental Procedures.” Thirty μl of G-100 solution (150 μg of protein) and hemocyte lysate (150 μg of protein) were used for reaction. The error bars represent triplicate in a single experiment.
Molecular Cloning of Holotrichia Hd-PGRP-1 and Hd-PGRP-2—To determine the whole amino acid sequences of the purified Hd-PGRP-1 and Hd-PGRP-2, we first determined two amino-terminal sequences and four partial amino acid sequences of Hd-PGRP-1 and Hd-PGRP-2. To isolate cDNA clones for the Hd-PGRP-1 and Hd-PGRP-2, we screened the cDNA library of *H. diomphalia* larvae with degenerated DNA probes made from the chemically determined partial amino acid sequences. The deduced amino acid sequences of Hd-PGRP-1 and Hd-PGRP-2 are shown in Fig. 3. Both *Holotrichia* Hd-PGRP-1 and Hd-PGRP-2 contain an open reading frame of 591 and 561 nucleotides corresponding to 197 and 187 amino acid residues with a calculated mass of 22,091.84 Da and 20,970.79 Da, respectively. During cDNA cloning of Hd-PGRP-1 and Hd-PGRP-2, we have obtained another Hd-PGRP homologue (Hd-PGRP-3) showing high sequence identity with Hd-PGRP-1 and Hd-PGRP-2, 53.3% and 69.5%, respectively. The cDNA sequences of Hd-PGRP-1, -2, and -3 have been submitted to DDBJ as accession numbers AB115774, AB115775, and AB115776, respectively. The two amino-terminal sequences and four partial amino acid sequences of the Hd-PGBP-1 and Hd-PGBP-2 perfectly matched the deduced amino acid sequences in the open reading frames (data not shown). Therefore, we conclude that these are cDNAs for Hd-PGRP-1 and Hd-PGRP-2.

It is known that insects and human PGRPs have a similar catalytic domain as bacteriophage T7 lysozyme (8, 9, 34). Among five amino acids (His-18, Tyr-47, His-123, Lys-129, and Cys-131) in the active site of T7 lysozyme, two residues (His-18 and Tyr-47) of T7 lysozyme are conserved in Hd-PGRP-1 and Hd-PGRP-2, but three residues (His-123, Lys-129, and Cys-131) of T7 lysozyme are not conserved in Hd-PGRP-1 and Hd-PGRP-2 (mutated to Ala, Thr, and Ser, respectively; boxes in Fig. 3). This result suggests that Hd-PGRP-1 and Hd-PGRP-2 are not likely to have N-acetylglucosamine 6-O-methyltransferase activity.

The amino acid sequence identities between Hd-PGRP-1 and Hd-PGRP-2 are 59.9%. As shown in Fig. 3, the homology between the J-Hd-PGRP-1, Hd-PGRP-2, and those in the National Center for Biotechnology Information data base showed that Hd-PGRP-1 is similar to *Bombyx mori* PGRP (Bm-PGRP, 42.2% identity) (9), *Trichoplusia ni* PGRP (Tn-PGRP, 43.1%) (8), *Drosophila melanogaster* PGRP-SA (Dm-PGRP, 45.7%) (35), human PGRP (39.1%) (8), mouse PGRP (40.2%) (8), rat PGRP (37.0%) (36), and bovine oligosaccharide-binding protein (bovine OBP, 39.2%) (37). Four cysteine and four tryptophan residues of Hd-PGRP-1 are conserved as in all insect PGRPs as well as in human (indicated by closed circles and closed reverse triangles in Fig. 3). Vertebrate PGRPs, such as human, mouse, and rat, have six cysteine residues compared with four residues in invertebrate PGRPs (indicated as closed diamonds).

Hd-PGRP-1 and Hd-PGRP-2 Are Localized in Plasma—To examine the localization of Hd-PGRP-1 and Hd-PGRP-2, we prepared fat body, plasma, and hemocyte lysate. As shown in Fig. 4, Hd-PGRP-1 and Hd-PGRP-2 were not detected in the hemocyte lysate or fat body (lanes 2 and 3), whereas a significant amount of protein was detected in the plasma (lane 1), indicating that Hd-PGRPs are localized in the plasma. Silkworm PGRP, *Drosophila* PGRP-SA and PGRP-SD were constitutively expressed in hemocytes, whereas inducible short PGRPs and PGRP-LB family expressed in the fat body (9, 35).

**Fig. 2.** SDS-PAGE pattern of the ESGC under reducing conditions (A), non-reducing conditions (B), and sequence comparison bands 7 and 8 with *Drosophila* PGRP-SA (C). A, lane 1, G-100 solution; lane 2, the hemocyte lysate; lane 3, 3 M NaCl elution solution; lane 4, ESGC. B, assignments of lanes are identical with A. C, the amino-terminal sequences of bands 7 and 8 are compared with that of *Drosophila* PGRP-SA (35).
To confirm the sugar recognition specificity of the recombinant Hd-PGRP-1 and Hd-PGRP-2 for 1,3-β-D-glucan and PGN, we prepared soluble PGN-coupled Sepharose CL-6B, 1,3-β-D-glucan-coupled Toyopearl, a control Sepharose CL-6B resin, and a control Toyopearl resin. Hd-PGRPs were detected both in the eluate solution of 1,3-β-D-glucan-resin and PGN-resin, but not in the control resins (Fig. 5B). Although the binding ability for Hd-PGRPs against glucan is apparently much weaker than for PGN, Hd-PGRPs specifically recognized both PGN and 1,3-β-D-glucan.

For quantitative analysis of Hd-PGRPs as 1,3-β-D-glucan pattern recognition proteins, we examined interactions between laminarioligosaccharides and Hd-PGRPs using a BIAcore system. The water-soluble laminarioligosaccharides were first derivatized with biotin and fixed onto the surface of a streptavidin-immobilized sensor chip. When the purified recombinant Hd-PGRP-1 was injected onto the laminaritetraose- and laminaripentaose-immobilized chip, it bound to those chips time- and dose-dependently. However, no specific binding could be detected using laminaribiose and laminaritriose chips (Fig. 6A). Under the same conditions, Hd-PGRP-2 did not bind to the laminaripentaose-immobilized chip (Fig. 6B). The binding parameters were obtained from the sensorgrams with different kinds of laminarioligosaccharides (Table I). The association constant (K_a) of the laminaripentaose with PGRP-I was even higher than that of laminaritetraose. However, K_a values of laminaritriose and laminaribiose cannot be determined because their binding to Hd-PGRP-1 is too low. These results support that Hd-PGRP-1 has a specific binding ability against 1,3-β-D-glucan.
1,3-β-D-glucan and a minimum structure for binding to Hd-PGRP-1 is laminaritetraose.

To obtain further information about minimum structural requirement of pattern recognition molecules to induce proPO activation, we tested the effects of laminarioligosaccharides on 1,3-β-D-glucan-dependent proPO activation. Laminariobiase and laminaritriose did not activate Holotrichia proPO system, whereas laminaritetraose with 4 glucose residues activated the proPO system (columns 4–6 in Fig. 7). When Hd-PGRP-1 or the mixture of Hd-PGRP-1 and Hd-PGRP-2 was added to the glucan-specific solution containing laminariheptaose and Ca²⁺, PO activity was increased ~1.5- and ~2-fold, respectively (lanes 9 and 11 in Fig. 7). However, Hd-PGRP-2 alone did not affect PO activity under the same conditions (lane 10). These results support that laminaritetraose is enough for 1,3-β-D-glucan-dependent proPO activation.

To further prove that Hd-PGRPs are 1,3-β-D-glucan pattern recognition proteins involved in the proPO system, we checked PO activity by using the purified recombinant Hd-PGRPs. When Hd-PGRP-1 (1 µg) was added to the glucan-specific solution in the presence of Ca²⁺, and 1,3-β-D-glucan, PO activity was increased ~1.5 fold after 20 min incubation compared with the control (column 8 in Fig. 8). But, addition of Hd-PGRP-2 did not result in an increase in PO activity under the same conditions (column 9), whereas a mixture of Hd-PGRP-1 and Hd-PGRP-2 increased the PO activity ~2.0-fold (column 11). Additionally, without 1,3-β-D-glucan, the increase of the PO activity was not induced in the presence of Hd-PGRP-1, or with Hd-PGRP-2 (columns 5 and 7). Under the same conditions, the PGN-dependent PO activity in the glucan-specific solution was not influenced by Hd-PGRP-1 or Hd-PGRP-2 (data not shown). The slightly increased PO activity by addition of Hd-PGRPs and soluble PGN might be attributed to a contamination of PGN with lipoteichoic acid. These results taken together suggest that Hd-PGRP-1 recognizes 1,3-β-D-glucan and then induce Holotrichia 1,3-β-D-glucan-dependent proPO activation. When both Hd-PGRP-1 and Hd-PGRP-2 were present during the proPO activation, the PO activity was more enhanced compared with Hd-PGRP-1 alone. The main difference between Hd-PGRP-1 and Hd-PGRP-2 is that Hd-PGRP-1 has six additional amino acid residues in the amino-terminal region (Fig. 3), which then suggests that this region in the amino terminus may be involved in correct binding of 1,3-β-D-glucan to this PGRP and that Hd-PGRP-2 lacking those 6 amino acids has a
low affinity for binding 1,3-β-D-glucan. The exact mechanism of this difference in PO activity remains to be determined.

To further explore whether Hd-PGRP-1 and Hd-PGRP-2 increase the amidase activity of the glucan-specific solution during 1,3-β-D-glucan-dependent proPO activation, we examined the change of amidase activity with trypsin substrate as previously described in Fig. 1B. As shown in Fig. 9, the amidase activity was increased by addition of Hd-PGRP-1, but not Hd-

---

**Fig. 7.** The ligand specificity of the purified Hd-PGRP-1 and Hd-PGRP-2 on the 1,3-β-D-glucan-dependent proPO activation system. The measurement of PO activity by *in vitro* reconstitution was described under “Experimental Procedures.” The error bars represent triplicate in a single experiment.

| Glucan specific soln. | + | + | + | + | + | + | + | + | + |
|-----------------------|---|---|---|---|---|---|---|---|---|
| Ca²⁺                  | - | + | + | - | + | + | + | + | + |
| 1,3-β-glucan          | - | - | + | - | + | + | + | + | + |
| biose                 | - | - | - | + | - | - | - | - | - |
| triose                | - | - | - | - | + | - | - | - | - |
| tetraose              | - | - | - | - | - | - | - | - | - |
| pentaose              | - | - | - | - | - | - | + | - | - |
| heptaose              | - | - | - | - | - | - | - | - | - |
| Hd-PGRP-1             | - | - | - | - | - | + | - | + | + |
| Hd-PGRP-2             | - | - | - | - | - | - | - | - | - |

---

**Fig. 8.** The effects of the purified Hd-PGRP-1 and Hd-PGRP-2 on the 1,3-β-D-glucan-dependent proPO activation system. PO activity was measured as described under “Experimental Procedures.” Fifteen µl of G-100 solution (75 µg of protein) and hemocyte lysate (75 µg of protein) were used for reaction.
PGRP-2, to the glucan-specific solution in the presence of 1,3-β-D-glucan and Ca$^{2+}$ (columns 12 and 13). These results are consistent with previous experiments in which Hd-PGRP-1, not Hd-PGRP-2, increased 1,3-β-D-glucan-dependent PO activity. These findings indicate that the recognition signal of Hd-PGRP-1 for 1,3-β-D-glucan might be transferred to the activation of serine protease zymogen, but Hd-PGRP-2 might involve as a cofactor for enhancing PO activity. The isolation and characterization of this serine protease zymogen remains to be performed.

**DISCUSSION**

We report here another function for the short form of PGRPs, which is that it can function as a 1,3-β-D-glucan pattern recognition protein and induce proPO activation in addition to binding PGN. The analysis of BIAcore experiments clearly supports that the short form Hd-PGRP-1 can bind laminaritetraose. By performing in vitro reconstitution experiments, it was found that Hd-PGRP-1 could induce 1,3-β-D-glucan-dependent PO activity. Söderhäll and colleagues (38) reported that the minimum structure for crayfish proPO activation system by laminarin was a laminaripentaose. Although the silkworm PGRP was the first identified protein that bound PGN and activated the proPO activation system, Söderhäll and colleagues (38) reported that the minimum structure for crayfish proPO activation system by laminarin was a laminaripentaose. Although the silkworm PGRP was the first identified protein that bound PGN and activated the proPO cascade in the hemolymph, Hd-PGRP-1, which has high sequence homology with silkworm PGRP, recognizes and binds 1,3-β-D-glucan and, after binding, elicits activation of the proPO activation system.

Regarding the function of Hd-PGRP-2, there is one possible explanation that a heterodimer of Hd-PGRP-1 and Hd-PGRP-2 induces more efficient 1,3-β-D-glucan-dependent proPO activation rather than Hd-PGRP-1 alone. Recently, the functional diversity of PGRP-LC family was reported by Werner et al. (39). Alternative splicing of Drosophila PGRP-LC results in that these PGRPs have different affinity to LPS and PGN. The simultaneous requirement of two splice forms (PGRP-LCa and -LCx) for the response to LPS suggests that the PGRPs may act as heterodimer or as higher multimers.

Recent data show that the recognition ligands of the PGRP family such as microbe cell wall components are quite diverse. For example, the long form PGRP-LC and PGRP-LE are absolutely required for the induction of antibacterial peptide genes through Imd/Relish pathway in response to Gram-negative bacteria infection in *Drosophila* (40, 41) or in *Drosophila* larvae (42). The activation of the Imd/Relish pathway by LPS as well as by Gram-negative bacteria raises the possibility that PGRP-LC recognizes LPS rather than PGN. Further support for this is the fact that PGRP-LC is involved in phagocytosis of Gram-negative bacteria but not Gram-positive bacteria (43). Also, it was reported that *Drosophila* Toll was activated by Gram-negative bacteria through a circulating PGRP-SA molecule (44). The crystal structure of *Drosophila* PGRP-LB demonstrates that poor conservation of surface residues at the active sites predicts a widely varying individual specificity of PGRPs for molecular patterns on microbial cell walls (45). Further these data suggest that the PGRP family has a principal role in sensing pathogens and that distinct PGRP molecules recognize different classes of microorganisms.

We isolated Hd-PGRPs by a soluble 1,3-β-D-glucan-immobilized Toyopearl 650M, a hydrophilic vinylpolymer-based resin. Hd-PGRPs bound tightly to 1,3-β-D-glucan column resin, but it could be eluted by 8 M urea. Interestingly, Hd-PGRPs were...
co-purified with six other proteins (Fig. 2A). The isolation and structural determination of these proteins will give us useful information for elucidation of proPO activation mechanism by 1,3-β-D-glucan. We reported the molecular relationship between three proPO-activating factors and two proPOs of the large beetle, *H. diophthalus* larvae. The two proPOs are activated by a two-step proteolysis reaction by two serine proteases and one serine protease homologue in the downstream part of proPO system (19). It is possible that pattern recognition proteins, such as GRP or PGRP, can make a complex with microbial cell components and proPO-activating serine protease(s), and then activated proPO-activating serine protease will convert proPO to active PO by a limited proteolysis, in a manner similar to *Drosophila* Toll, which is activated through a cleaved form of the cytokine Spatzle by an easter serine protease (46).

Recently, it was reported that *Drosophila* PGRP-SC1B has a N-acetylmuramyl-L-alanine amidase activity and that it might have a scavenger function (47). Also, *Drosophila* PGRP-LB has been shown to have bacterial cell wall lytic activity (48). The two proPOs are activated by an easter serine protease (46). The isolation and characterization of the cytokine Spatzle by an easter serine protease (46). The isolation and characterization of the cytokine Spatzle by an easter serine protease (46).
Peptidoglycan Recognition Proteins Involved in 1,3-β-D-Glucan-dependent Prophenoloxidase Activation System of Insect
Mi Hee Lee, Tsukasa Osaki, Joo Young Lee, Min Ji Baek, Rong Zhang, Ji Won Park, Shun-ichiro Kawabata, Kenneth Söderhäll and Bok Luel Lee

J. Biol. Chem. 2004, 279:3218-3227.
doi: 10.1074/jbc.M309821200 originally published online October 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309821200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 24 of which can be accessed free at http://www.jbc.org/content/279/5/3218.full.html#ref-list-1