Characterization and Properties of a 1,3-β-D-Glucan Pattern Recognition Protein of Tenebrio molitor Larvae That Is Specifically Degraded by Serine Protease during Prophenoloxidase Activation*

Received for publication, July 11, 2003, and in revised form, August 15, 2003
Published, JBC Papers in Press, August 15, 2003, DOI 10.1074/jbc.M307475200

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Although many different pattern recognition receptors recognizing peptidoglycan and 1,3-β-D-glucan have been identified in vertebrates and insects, the molecular mechanism of these molecules in the pattern recognition and subsequent signaling is largely unknown. To gain insights into the action mechanism of 1,3-β-D-glucan pattern recognition protein in the insect prophenoloxidase (proPO) activation system, we purified a 53-kDa 1,3-β-D-glucan recognition protein (Tm-GRP) to homogeneity from the hemolymph of the mealworm, Tenebrio molitor, by using a 1,3-β-D-glucan affinity column. The purified protein specifically bound to 1,3-β-D-glucan but not to peptidoglycan. Subsequent molecular cloning revealed that Tm-GRP contains a region with close sequence similarity to bacterial glucanases. Strikingly, two catalytically important residues in glucanases are replaced with other nonhomologous amino acids in Tm-GRP. The finding suggests that Tm-GRP has evolved from an ancestral gene of glucanases but retained only the ability to recognize 1,3-β-D-glucan. A Western blot analysis of the protein level of endogenous Tm-GRP showed that the protein was specifically degraded following the activation of proPO with 1,3-β-D-glucan and calcium ion. The degradation was significantly retarded by the addition of serine protease inhibitors but not by cysteine or acidic protease inhibitor. These results suggest that 1,3-β-D-glucan pattern recognition protein is specifically degraded by serine protease(s) during proPO activation, and we propose that this degradation is an important regulatory mechanism of the activation of the proPO system.

The innate immune system is a host defense mechanism that is evolutionarily conserved from plants to humans (1, 2) and is mainly involved in the recognition and control of the early stage of infection in all animals. 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),1 peptidoglycan (PGN), 1,3-β-D-glucan, and mannan (referred to as pathogen-associated molecular patterns). Upon recognition, these receptors activate distinct signaling cascades that regulate specific gene expression programs 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).

The activation of the prophenoloxidase (proPO) cascade is a major innate immune reaction in invertebrates triggered by elicitors derived from microbial cell walls such as LPS, PGN, and glucan (7–9). The proPO activation pathway, like the vertebrate complement system, is a proteolytic cascade containing several serine proteases and their inhibitors and terminates with thezymogen, proPO. Microbial carbohydrates such as LPS, PGN, or 1,3-β-D-glucan will first react with pattern recognition proteins, which then will induce activation of several serine proteases within the proPO system (7–12). It is known that one of the serine proteases in the cascade, which is named proPO-activating enzyme or factor, will cleave proPO to generate the active enzyme, phenoloxidase (PO) (13–16). This enzyme can produce 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 (17). Determining the molecular mechanism by which pattern recognition molecules differentiate non-self from self and transduce signals that stimulate defense responses is a key for understanding the ways in which innate immune systems are regulated. Many reports have been published about the invertebrate’s proPO and its activation mechanism (10–22). One important issue is the early events of the proPO activation system and more specifically how pattern recognition mole-

1 The abbreviations and trivial name used are: LPS, lipopolysaccharide; proPO, prophenoloxidase; Tm-GRP, T. molitor β-1,3-β-glucan recognition protein; PGN, peptidoglycan; PO, phenoloxidase; PGR, peptidoglycan recognition proteins; GRP, 1,3-β-D-glucan recognition protein; GNBP, Gram-negative bacteria binding protein; LGBP, LPS, and 1,3-β-D-glucan-binding proteins; ELSG, eluate solution from 1,3-β-D-glucan-immobilized column; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; p-APMSF, p-amidino-naphthyl methane sulfonfl fluoride hydrochloride; Z, benzoxycarbonyl; Boc, butoxycarbonyl; Suc, succinyl; MCA, 4-methyl-coumaryl-7-amide; E-64, 1-trans-epoxysuccinyl-leucylamido(4-guanidino)butane.
cules induce activation of the proPO system in response to pathogenic microbial infection.

Although several pattern recognition proteins, such as PGN recognition proteins (PGRPs) (23–31), 1.3-β-D-glucan recognition protein (GRP) (11, 12, 32–34), Gram-negative bacteria-binding proteins (GNBPs) (35–38), and LPS and 1.3-β-D-glucan-binding proteins (LGBPs) (10, 39), have been identified in invertebrates, the molecular mechanism and structural basis of microbial recognition are poorly defined. We were able to reconstitute in vitro the downstream components of the proPO cascade using biochemically purified compounds from the large insect, Holotrichia diomphalia larvae (18). Although we have determined that the downstream part of proPO system is regulated by two easter-type serine proteases and a maquase-type serine protease homologue and involves two-step limited proteolysis for showing PO activity (18), the upstream part of the proPO cascade is still poorly understood. Also, it remains to be elucidated how the connection between the upstream and downstream part of the proPO cascade is organized and activated. A hypothesis is that the pattern recognition molecules, such as PGRPs, GRPs, GNBPs, and LGBPs, will make a complex with the proPO-activating enzyme(s) and microbial cell wall components, and then activated proPO-activating enzyme(s) will convert proPO to active PO by a limited proteolysis.

We have undertaken a task to purify 1.3-β-D-glucan-binding protein to understand the upstream part of the proPO cascade in the larvae of mealworm, Tenebrio molitor. Here we present the isolation, molecular cloning, and biological functions of a new 1.3-β-D-glucan pattern recognition protein.

EXPERIMENTAL PROCEDURES

Animals—T. molitor larvae (mealworm) were maintained on a laboratory bench in terraria containing wheat bran. Vegetables were placed on top of the bran to provide water. Hemolymph and hemocytes were collected as previously described (19). Briefly, to harvest the hemolymph, larvae were injected with 50 μl of a modified anti-coagulation buffer (30 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 5.5, buffer A) using a 25-gauge needle. The tail of each larva was cut off using a fine pair of scissors, and the extruding hemolymph was placed in a test tube on ice. The collected crude hemolymph was centrifuged at 203,000 × g for 4 h at 4 °C. The supernatant was then stored at −80 °C until use.

Assay of PO Activity—An assay of PO was carried out according to our previously published method (13). Briefly, to measure PO activity, 30 μl of crude hemolymph (150 μg of proteins) or fractionated solution from column chromatography was preincubated in 85 μl of 20 mM Tris-HCl buffer (pH 8.0) containing 1 μM of 1.3-β-D-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 PO activity was defined as the amount of enzyme causing an increase in absorbance of 0.1 at 520 nm per 10-min incubation (A520/min).

To examine the effects of the eluate solution from the 1.3-β-D-glucan affinity column (referred to as ELSG) on PO activity, 1.3-β-D-glucan-binding protein-excluded hemolymph was obtained by passing Tenebrio hemolymph through the 1.3-β-D-glucan-immobilized column. The bound proteins on the 1.3-β-D-glucan column were eluted with buffer A containing 8 mM urea, and then urea was immediately excluded by repeated ultrafiltrations (YM10 membrane; Amicon) with buffer A. To reconstitute the 1.3-β-D-glucan-dependent PO activity, the urea-excluded eluate solution and 1.3-β-D-glucan-binding protein-excluded hemolymph was incubated in the presence of 1.3-β-D-glucan and Ca2+, and then PO activity was examined. To check the effects of the purified T. molitor 1.3-β-D-glucan-binding Protein (Tm-GRP) on the Tenebrio proPO system, the purified native Tm-GRP (0.5 μg) was incubated with crude hemolymph (250 μg) containing the proPO system and the ELSG or control column solution (250 μg) of the 1.3-β-D-glucan affinity column at 30 °C for 30 min in the presence of Ca2+ and 1.3-β-D-glucan as described above.

Preparation of 1.3-β-D-Glucan Affinity Column by Using AF-Amino Toyopearl 650 M Resin—A 1,3-β-D-glucan affinity column was prepared as previously described (41). Briefly, 3 ml of in soluble 1.3-β-D-glucan (curdlan; Wako) stock solution (500 mg of curdlan was dissolved in 15 ml of 1 N NaOH solution) was added to 20 ml of 0.2 M K2HPO4, and pH was adjusted to 8.4 with 3 M HCl. 2.5 g of suction-dried AF-Amino Toyopearl 650 M was suspended in the soluble curdlan solution prepared with 10 N NaOH. After the addition of 1 g of NaN3BH4, the suspension was incubated at 60 °C overnight. The gel was then acetylated to block remaining free amino groups by incubating with 8 ml of 0.2 M sodium acetate and 4 ml of acetic anhydride on ice for 30 min followed by the addition of an additional 4 ml of acetic anhydride and then incubation for 30 min at room temperature. Finally, the resin was washed with 150 ml of 0.1 M NaOH, 150 ml of 1 M Tris-HCl (pH 8.0) and finally with a sufficient amount of distilled water. As a control, the resin was treated with the same procedure described above but without curdlan. The coupled 1.3-β-D-glucan to resins were quantified by the sulfuric acid-phenol method (42). The resins that bound more than 3 μg of glucose/mg of resin were used for purification of Tenebrio 1.3-β-D-glucan-binding protein.

Purification of the Denatured Tenebrio Tm-GRP.—To purify lymph proteins that can recognize 1.3-β-D-glucan from the hemolymph of T. molitor larvae, 50 ml of hemolymph solution (300 mg of proteins) was collected after ultracentrifugation at 203,000 × g for 4 h at 4 °C and then filtered with 0.45-μm Gelman polyvinylidene difluoride membrane. The obtained supernatant solution was applied to a 1.3-β-D-glucan-immobilized Toyopearl 650 M column (2.5 × 5 cm) equilibrated with buffer A at 0.6 ml/min. After washing the column with buffer A until no absorbance at 280 nm, bound proteins were eluted at a rate of 0.5 ml/min with buffer A containing 8 M urea and then analyzed by SDS-PAGE under reducing and nonreducing conditions. The major 53-kDa band was cut out from the gel and was then extracted by electrosolution with 250 mA for 3 h at 4 °C according to the manufacturer’s instructions. Finally, the purity of the 53-kDa was checked by SDS-PAGE under reducing conditions. On the control column (2.5 × 5 cm), 20 ml of hemolymph was loaded to the column, which had been preequilibrated with buffer A at 0.6 ml/min, and bound proteins were eluted with the same method as described above.

To determine the partial amino acid sequences of the purified 53-kDa protein (the purified protein was reduced, alkylated, and digested with 2 μg of lysylendopeptidase at 37 °C for 13 h. The digested peptides were separated by HPLC on a C18 reverse phase column (Gilion) with a linear gradient between 0.05% trifluoroacetic acid in water and 0.052% trifluoroacetic acid in 80% acetonitrile (18). The amino-terminal amino acid sequence of the purified 53-kDa protein and the amino acid sequences of internal peptides from HPLC were determined on an Applied Biosystem Procise automated gas phase sequencer (43).

Purification of the Native Tm-GRP—After ultracentrifugation of 100 ml of crude hemolymph, the resulting supernatant was loaded to a control Toyopearl 650 M column (2.5 × 5 cm), which had been pre-equilibrated with buffer A containing 8 M urea, at a rate of 0.6 ml/min. The flow-through solution from the control column was loaded again to a 1.3-β-D-glucan-coupled Toyopearl 650 M column, which also pre-equilibrated with buffer A at the same flow rate as the control Toyopearl column. After washing the column with buffer A until absorbance at 280 nm was zero, bound proteins were eluted with 50 ml of buffer A containing 8 M urea at 0.5 ml/min, and eluted proteins were analyzed on SDS-PAGE under nonreducing conditions. The 53-kDa Tm-GRP was cut out from the gels. After transferring the gel slices to a new Eppendorf tube, the proteins on crushed gels were carefully extracted with 200 μl of buffer A containing 8 M urea at 40 °C for 30 min. After loading the extraction solution to plasmaid extraction microspin column (Qiagen), the column was centrifuged at 30 min at 4 °C followed by elution with buffer B at 0.2 ml/min. The purified native Tm-GRP was confirmed by SDS-PAGE under reducing and nonreducing protein conditions.

cDNA Cloning and Nucleotide Sequencing of 53-kDa Tm-GRP—A cDNA library from T. molitor larvae was constructed with a previously described method (13) using a ZAP-cDNA synthesis kit (Stratagene). Seven partial amino acid sequences of the purified 53-kDa protein were determined. Among them, a DNA oligonucleotide corresponding to PD-DGAGTGAACGTGCTCGGTGCCTGTATTGTTCAATGA was synthesized, and it was labeled with r-32PdATP by a previously described method (44). For the initial screening, ~120,000 recombinants of T. molitor larvae cDNA library were used. The membranes were prehybridized at 65 °C for 1 h in 5× SSC (750 mM NaCl, 75 mM sodium acetate) and then hybridized with the probe at 42 °C overnight. The membranes were washed with 0.1× SSC/0.1% SDS at 65 °C for 30 min followed by exposure to X-ray film after autoradiography.
citrate, pH 7.0, 5× Denhardt's solution (100× Denhardt's solution: 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll, and 2% (w/v) polyvinylpyrrolidone), 100 µg/ml salmon sperm DNA, and 0.5% SDS. The membranes were then hybridized at 65 °C for 12 h in the same solution as during prehybridization. Five hybridization-positive clones were obtained, and two of them were found to contain an 882-base pair fragment of Tm-GRP cDNA.

To obtain the full cDNA of Tm-GRP, the cDNA library from T. molitor larvae was screened once again with a 770-base pair fragment prepared by the random primer method and labeled with [α-32P]dCTP. Plaques that hybridized to the probe were purified to homogeneity and subcloned by in vivo excision of pBluescript plasmids. The positive clone with the longest cDNA insertion was sequenced using vector primers with an Applied Biosystems PRISM dye terminator cycle sequencing ready reaction kit (PerkinElmer Life Sciences). The amino acid sequence of the 53-kDa Tm-GRP was compared with the protein sequence data base of the National Center for Biotechnology Information (NCBI) using the Genetyx system (Software Development Co., Ltd., Tokyo).

Antibody and Immunoblotting—An antibody against purified Tm-GRP was raised by injecting 10 µg of the purified protein into a male albino rabbit with complete Freund's adjuvant and giving two booster injections with the same amount of protein 7 and 14 days later (45). The resulting antibody was affinity-purified as previously described (46). For immunoblotting, the proteins separated on SDS-PAGE were transferred electrothermally to a polyvinylidene difluoride membrane, and the membrane was blocked by immersion in 5% skimmed milk solution containing 1% horse serum for 12 h. The membrane was then transferred to rinse solution I (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20, and 2.5% skimmed milk) containing the affinity-purified antibody against Tm-GRP (50 ng/ml) and incubated at 4 °C for 2 h. The bound antibody was identified using the ECL Western blotting reagent kit (Amersham Biosciences).

Determination of Tm-GRP Localization—Hemolymph was obtained from larvae and centrifuged at 3,000 rpm at 4 °C for 10 min. The supernatant was used as a plasma sample. The precipitated hemocytes were washed with 500 µl of buffer A and suspended again with 500 µl of buffer B, sonicated for 15 s at 4 °C, and then centrifuged at 15,000 rpm for 30 min (4°C) to collect the supernatant. The supernatant was used as a source of hemocyte lysate. The soluble proteins were precipitated with trichloroacetic acid and subjected to SDS-PAGE and then immunoblotting with the affinity-purified antibody raised against the purified 53-kDa Tm-GRP.

Western Blot Analysis of 53-kDa Tm-GRP during proPO Activation—To follow the change of the amount of endogenous Tm-GRP during proPO activation, we first measured β-1,3-glucan-dependent PO activity by incubating the pass-through solution from the glucan column with the ELGS or incubating the purified 53-kDa Tm-GRP with the crude hemolymph both in the presence of 1.3-β-glucan and Ca2+. The reaction mixtures were precipitated with trichloroacetic acid at various time intervals. After performance of SDS-PAGE, the amount of 53-kDa Tm-GRP was examined by Western blot analysis using the purified Tm-GRP antibody.

Effects of Protease Inhibitors on Degradation of Tm-GRP and Tenebrio proPO System—Four kinds of protease inhibitors (p-nitroguanidine benzoxa, p-amidinophenyl methane sulfonly fluoride hydrochloride (p-APMSF), t-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), and pepstatin A) were used to reveal whether these inhibitors affected proPO activation, we first measured β-1,3-glucan-dependent PO activity by incubating the pass-through solution from the glucan column with the ELGS or incubating the purified 53-kDa Tm-GRP with the crude hemolymph both in the presence of 1.3-β-glucan and Ca2+. The reaction mixtures were precipitated with trichloroacetic acid at various time intervals. After performance of SDS-PAGE, the amount of 53-kDa Tm-GRP was examined by Western blot analysis using the purified Tm-GRP antibody.

RESULTS

Identification of a 1.3-β-D-Glucan Pattern Recognition Protein from T. molitor Hemolymph—We first examined the 1.3-β-D-glucan-dependent PO activity by using T. molitor crude hemolymph. As shown in Fig. 1, crude hemolymph exhibited the most rapid increase in PO activity in the presence of 1.3-β-D-glucan and Ca2+. Under the same conditions, PO activity was not observed with only 1.3-β-D-glucan. However, Ca2+- alone activated proPO system, but more slowly than that with both 1.3-β-D-glucan and Ca2+. These results suggest that Tenebrio hemolymph contains all necessary components for 1.3-β-D-glucan-dependent proPO activation, such as Tm-GRPs(s), prophenoloxidase-activating factors, and proPO.

To purify 1.3-β-D-glucan pattern recognition protein(s) from T. molitor larval hemolymph, we prepared a 1,3-β-D-glucan-coupled Toyopearl resin and 1,3-β-D-glucan-coupled Toyopearl resin or 10 µl of soluble PGN-coupled Sepharose resin for 30 min at 4 °C. After collecting the supernatant by centrifugation at 13,000 rpm for 30 s, the pellet containing the resin was washed with 600 µl of buffer A four times. The bound proteins were eluted with 500 µl of buffer A containing 8 M urea after incubation at 4 °C for 30 min. The supernatant and the eluted solution from resins were precipitated with trichloroacetic acid, and then the amount of Tm-GRP was estimated by Western blot with Tm-GRP antibody.

To prove that ELSG contained Tm-GRP(s), we checked PO activity in the crude hemolymph of T. molitor larvae. PO activity was measured as described under "Experimental Procedures." 30 µl of crude hemolymph (150 µg) was incubated with Ca2+ and 1.3-β-D-glucan (closed squares), Ca2+ only (closed circles), 1.3-β-D-glucan only (closed triangles), and neither Ca2+ nor 1.3-β-D-glucan (closed diamonds).

![Fig. 1. PO activity in the crude hemolymph of T. molitor larvae. PO activity was measured as described under "Experimental Procedures." 30 µl of crude hemolymph (150 µg) was incubated with Ca2+ and 1.3-β-D-glucan (closed squares), Ca2+ only (closed circles), 1.3-β-D-glucan only (closed triangles), and neither Ca2+ nor 1.3-β-D-glucan (closed diamonds).](http://www.jbc.org/Downloaded from http://www.jbc.org/)
1,3-β-D-glucan and Ca\(^{2+}\) (column 6). Under the same conditions, pass-through solution from the control column clearly showed 1,3-β-D-glucan-dependent PO activity (column 3). These results suggest that Tm-GRP(s) in crude hemolymph is removed from hemolymph by passing through a 1,3-β-D-glucan affinity column.

To confirm that it is possible to recover 1,3-β-D-glucan-dependent PO activity, the pass-through fraction from the glucan column was incubated with the ELSG in which the urea had been immediately removed by dialysis with buffer A, and then in vitro reconstitution experiments were performed. As shown in Fig. 3A, PO activity was recovered when the ELSG was incubated with the pass-through solution of the 1,3-β-D-glucan affinity column and control column were measured in the presence of Ca\(^{2+}\) and 1,3-β-D-glucan.

Previously, we have reported that the proPO activation system of H. diomphalia larvae is activated by two easter-type serine proteases and one masquerade-like serine protease (18). To explore which protease was present in the ELSG, we measured the amidase activity by using commercially available peptidyl-NH-Mec substrates. As shown in Fig. 3B, trypsin and α-thrombin substrates were specifically hydrolyzed when the ELSG was incubated with these peptidyl-NH-Mec substrates (columns 2 and 3). However, it did not hydrolyze substrates for chymotrypsin, elastase, and cathepsin B (columns 4, 5, and 6). Furthermore, the amidase activity of trypsin substrate in the ELSG could also be inhibited by specific serine protease inhibitors, p-AMPSF and p-nitroguanidino benzoate (data not shown). These data suggest that serine protease(s) coexists with Tm-GRP(s) in the ELSG.

When the ELSG was analyzed by SDS-PAGE under reducing condition, a 53-kDa protein was found to be mostly enriched compared with the crude hemolymph (indicated by the arrow in Fig. 2A). Therefore, we purified the 53-kDa protein by repeated electroelution, and it was purified to homogeneity (Fig. 2A). The amino-terminal sequence of the Tm-GRP was blocked when analyzed by Edman degradation, and seven partial amino acid sequences derived from the lysylendopeptidase-treated Tm-GRP (EGDILYYWTYVDYFDG, VNERVCAGEQIFVLD, FADKPDYEFVFYRAG, VNLAPACTGVHGSIE, GDWIIPELYLN, FIVWKPDQITMMVD, and EMYLVLGGVGG) were obtained as follows. When these seven amino acid sequences of the purified 53-kDa protein were compared with NCBI data, four sequences showed sequence homology with those of Bombyx mori GRP (11) and Manduca sexta GRP (12) (data not shown). This result suggests that the purified 53-kDa protein is likely to be a Tenebrio GRP.

**cDNA Cloning and Nucleotide Sequence Analysis of Tm-GRP**—The cloned cDNA of Tm-GRP contained an open reading frame of 1443 nucleotides corresponding to 481 amino acid residues with a predicted mass of 54,034.2 Da. The seven chemically determined partial amino acid sequences of the purified 53-kDa protein were compared with the open reading frame, and seven partial amino acid sequences derived from the lysylendopeptidase-treated Tm-GRP (EGDILYYWTYVDYFDG, VNERVCAGEQIFVLD, FADKPDYEFVFYRAG, VNLAPACTGVHGSIE, GDWIIPELYLN, FIVWKPDQITMMVD, and EMYLVLGGVGG) were obtained as follows. When these seven amino acid sequences of the purified 53-kDa protein were compared with NCBI data, four sequences showed sequence homology with those of Bombyx mori GRP (11) and Manduca sexta GRP (12) (data not shown). This result suggests that the purified 53-kDa protein is likely to be a Tenebrio GRP.

**Experimental Procedures.**—The measurement of PO activity in vitro reconstitution is described under “Experimental Procedures.” The amidase activity was examined by using the following substrates. Column 1, 8 M urea elution buffer only; column 2, Boc-Phe-Ser-Arg-MCA; column 3, Boc-Val-Pro-Arg-MCA; column 4, Suc-Ala-Pro-Ala-MCA; column 5, Suc-Leu-Leu-Val-Tyr-MCA; column 6, Z-Phe-Arg-MCA.
with known proteins revealed the closest match with B. mori GRP (Bm-GRP; 42% identity) (11) and M. sexta GRP (Ms-GRP; 40% identity) (12) as shown in Fig. 4. These proteins contain a similar domain with those of Bm-GRP (42% identity) (11) and GRP (Bm-GRP) similar domain with those of Ms-GRP (40% identity) (12) as shown in Fig. 4. These proteins contain a

Localization of Tm-GRP—To examine the localization of Tm-GRP, we prepared fat body, plasma, hemocyte lysate, and hemolymph from T. molitor larvae as previously described (46). As shown in Fig. 6, no Tm-GRP was detected in the fat body or hemocyte lysate (lanes 3 and 4), but a significant amount of Tm-GRP was detected in plasma and hemolymph (lanes 1 and 2). This result strongly suggests that Tm-GRP is not likely to have glucanase activity.

Purification of Native Tm-GRP and in Vitro Reconstitution Experiments—The procedures of purification of the native Tm-GRP are shown in Fig. 7A. 100 ml of hemolymph was loaded to the control Toyopearl for excluding nonspecific binding proteins to the Toyopearl resin. The pass-through solution of control Toyopearl column still showing 1,3-β-glucan-dependent PO activity was loaded again to a 1,3-β-glucan-immobilized Toyopearl 650 M column. To purify Tm-GRP, the ELSG was analyzed by SDS-PAGE under nonreducing conditions, and then the Tm-GRP band was cut out. After extracting with buffer A containing 8 M urea, the eluted solution was immediately loaded to a Superdex 200 FPLC to remove urea and SDS. The purity of Tm-GRP was examined by SDS-PAGE under reducing and nonreducing conditions. 20 μg of Tm-GRP can be obtained from 100 ml of hemolymph. As shown in Fig. 7B, 100 ml of hemolymph was loaded to a 1,3-β-glucan-dependent PO activity was loaded again to a 1,3-β-glucan-immobilized Toyopearl 650 M column. To purify Tm-GRP, the ELSG was analyzed by SDS-PAGE under nonreducing conditions, and then the Tm-GRP band was cut out. After extracting with buffer A containing 8 M urea, the eluted solution was immediately loaded to a Superdex 200 FPLC to remove urea and SDS. The purity of Tm-GRP was examined by SDS-PAGE under reducing and nonreducing conditions. 20 μg of Tm-GRP can be obtained from 100 ml of hemolymph. As shown in Fig. 7B, 100 ml of hemolymph was loaded to a 1,3-β-glucan-dependent PO activity was loaded again to a 1,3-β-glucan-immobilized Toyopearl 650 M column. To purify Tm-GRP, the ELSG was analyzed by SDS-PAGE under nonreducing conditions, and then the Tm-GRP band was cut out. After extracting with buffer A containing 8 M urea, the eluted solution was immediately loaded to a Superdex 200 FPLC to remove urea and SDS. The purity of Tm-GRP was examined by SDS-PAGE under reducing and nonreducing conditions. 20 μg of Tm-GRP can be obtained from 100 ml of hemolymph. As shown in Fig. 7B,
Tm-GRP had a mass of 53 kDa under reducing conditions and 70 kDa under nonreducing conditions.

To examine the biological functions of the purified 53-kDa Tm-GRP in the Tenebrio proPO activation system, we performed in vitro reconstitution experiments by using the purified native Tm-GRP (C). The purified native Tm-GRP was incubated with the crude hemolymph or pass-through solution of 1,3-β-D-glucan-coupled column in presence of Ca2+ and 1,3-β-D-glucan at 30 °C for 30 min.

FIG. 7. Purification scheme of the native Tm-GRP (A), SDS-PAGE pattern of the purified native Tm-GRP (B), and in vitro reconstitution experiments by using the purified native Tm-GRP (C). The purified native Tm-GRP was incubated with the crude hemolymph or pass-through solution of 1,3-β-D-glucan-coupled column in presence of Ca2+ and 1,3-β-D-glucan at 30 °C for 30 min.

Mr. T. was not induced (lanes 3 and 4), and columns 3/H9252 in vitro PGN, we performed in vitro reconstitution experiments by using the purified native Tm-GRP. The purified native Tm-GRP was added to crude hemolymph. As shown in Fig. 9A, the amount of Tm-GRP in the crude hemolymph was gradually decreased when PO activity was increased. This experiment suggests that Tm-GRP may be degraded when 1,3-β-D-glucan and Ca2+ are used to activate the Tenebrio proPO system. To further explore whether Tm-GRP was degraded during proPO activation, we added native purified Tm-GRP (1 μg) to the crude hemolymph. As shown in Fig. 9B, Tm-GRP was more quickly degraded compared with that occurring in the crude hemolymph only (lanes 4 in Fig. 9, A and B). These results suggest that larger quantities of Tm-GRP induce higher PO activity, and as a result Tm-GRP is degraded more quickly than that occurring in the crude hemolymph.

To examine what type of protease can degrade Tm-GRP, the effects of protease inhibitors on this degradation were examined during proPO activation. As shown in Fig. 9C, p-APMSF, which is a specific serine protease inhibitor, could inhibit degradation of Tm-GRP even in the presence of exogenous Tm-GRP for 30 min (lane 5 in Fig. 9C). However, when E-64 (cysteine protease inhibitor) and pepstatin (acidic protease inhibitor) were added to crude hemolymph or the mixture of hemolymph and the purified Tm-GRP, Tm-GRP was degraded at the same rate as under control conditions (lanes 3 and 5 in Fig. 9D). Under the same conditions, the PO activity was not influenced by these two inhibitors (columns 5 and 6 in Fig. 9E). These results suggest that Tm-GRP was specifically degraded by serine protease(s) during proPO activation in T. molitor larvae.

DISCUSSION

We report here the purification, molecular cloning, and biochemical characterization of Tm-GRP from the hemolymph of T. molitor larvae. In this paper, we have demonstrated two novel findings regarding a glucan-binding protein from an invertebrate. One is that this protein is degraded by a serine protease(s) when PO activity is triggered by 1,3-β-D-glucan and Ca2+. The other is that the purified homogenous Tm-GRP is not sufficient for inducing 1,3-β-D-glucan-dependent proPO activation in the Tenebrio proPO system, suggesting that additional protein(s) is necessary.

We isolated Tm-GRP by a soluble 1,3-β-D-glucan-immobilized Toyopearl 650 M, a hydrophilic vinylpolymer-based resin. Tm-GRP bound so tightly to 1,3-β-D-glucan-immobilized Toyopearl resin that it could be eluted by 8 M urea only. Interestingly, the eluate solution from 1,3-β-D-glucan Toyopearl column showed a strong amidase activity against serine protease synthetic substrates but not in eluate solution from control Toyopearl resin. The whole amino acid sequence of Tm-GRP deduced from the cloned cDNA sequence indicates that the carboxyl-terminal domain of Tm-GRP has a similar domain to...
of protease inhibitors. Column 1 only incubated for 30 min; lane 2 column 2, the purified Tm-GRP was added to the 4 lane 2 hemolymph only at 0 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 0.3 μg of Tm-GRP only. Column 2, 30 min after the addition of the purified Tm-GRP and p-APMSF to hemolymph, lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 0.3 μg of Tm-GRP only. C, the effects of p-APMSF on Tm-GRP degradation. Lane 1, crude hemolymph only at 0 min; lane 2, 0 min after the addition of the purified Tm-GRP and p-APMSF; lane 3, after 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 0.3 μg of Tm-GRP only. D, the same amounts of protease inhibitors, p-APMSF, E-64, and pepstatin, were treated and analyzed after 30 min as described in C. Lane 1, crude hemolymph was only incubated for 30 min; lane 2, after 30-min incubation with hemolymph and the purified Tm-GRP 1; lane 3, E-64; lane 4, p-APMSF; lane 5, pepstatin; lane 6, Tm-GRP only. E, the measurement of PO activity is described under “Experimental Procedures” in the presence of three kinds of protease inhibitors. Column 1, only crude hemolymph was incubated for 30 min in the presence of Ca2+ and 1,3-β-D-glucan at 30 °C; column 2, the mixture of hemolymph and the purified Tm-GRP was incubated for 30 min; column 3, p-APMSF was added to the column 2 solution; column 4, the purified Tm-GRP was added to the column 2 solution; column 5, E-64 was added to the column 2 solution; column 6, pepstatin was added to the column 2 solution.

Fig. 9. Amount of Tm-GRP during proPO activation in the presence of Tm-GRP or protease inhibitors. A, after incubation of 50 μg of hemolymph with Ca2+ and 1,3-β-D-glucan at different time intervals, proteins were precipitated with trichloroacetic acid and subjected to SDS-PAGE. Tm-GRP was detected by Western blot against Tm-GRP antibody. Lane 1, 0 min; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 40 min; lane 6, 60 min. B, after 0.3 μg of Tm-GRP was added to 50 μg of hemolymph, samples were prepared as described for A. Lane 1, crude hemolymph only at 0 min; lane 2, 0 min after 0.3 μg of Tm-GRP addition; lane 3, after 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 0.3 μg of Tm-GRP only. C, the effects of p-APMSF on Tm-GRP degradation. Lane 1, crude hemolymph only at 0 min; lane 2, 0 min after the addition of the purified Tm-GRP and p-APMSF; lane 3, after 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 0.3 μg of Tm-GRP only. D, the same amounts of protease inhibitors, p-APMSF, E-64, and pepstatin, were treated and analyzed after 30 min as described in C. Lane 1, crude hemolymph was only incubated for 30 min; lane 2, after 30-min incubation with hemolymph and the purified Tm-GRP 1; lane 3, E-64; lane 4, p-APMSF; lane 5, pepstatin; lane 6, Tm-GRP only. E, the measurement of PO activity is described under “Experimental Procedures” in the presence of three kinds of protease inhibitors. Column 1, only crude hemolymph was incubated for 30 min in the presence of Ca2+ and 1,3-β-D-glucan at 30 °C; column 2, the mixture of hemolymph and the purified Tm-GRP was incubated for 30 min; column 3, p-APMSF was added to the column 2 solution; column 4, the purified Tm-GRP was added to the column 2 solution; column 5, E-64 was added to the column 2 solution; column 6, pepstatin was added to the column 2 solution.

that of bacterial β-1,3-glucanase or β-1,3- and β-1,4-glucanase (48) and sea urchin β-1,3-glucanase (49) (Fig. 5). Two catalytic Glu residues, conserved in the active site of the bacterial glucanases, are replaced with Gln and Phe residues in Tm-GRP, respectively. Interestingly, glucan binding proteins from silkworm, tobacco hornworm, fall webworm, fly, and mealworm are all replaced with other residues, suggesting that these GRPs do not have glucanase enzyme activity. However, other 1,3-β-D-glucan recognition or binding proteins, such as factor-G of horseshoe crab (33), coelomic cytolytic factor 1 of earthworm (39), LGBP of crayfish (10), and GNBPs of mosquito (38) and silkworm (36), have all conserved Glu residues in the active site as the bacterial glucanase (Fig. 5). Whether these differences between these two groups of glucan-binding proteins have any importance for the recognition of pattern molecules such as, for example, affinity constant differences or any other functional difference remains to be shown.

It is well known that pathogenic microbial infections in insects and other invertebrates trigger the activation of the proPO system (8). Several groups have determined the biological functions of proPO-activating factors and proPOs, suggesting that the proPO system is activated by a serine protease cascade and activated PO can synthesize melanin pigments for inhibiting spread of a microbial infection or for wound healing (8, 9). However, a continuous activation of the proPO cascade, which could be harmful for the host organisms, might be well regulated by pattern recognition receptors, serpins, or proPO-activating factors. The early events of the proPO system consist...
of two parts; one is the recognition reaction between invading pathogens and pattern recognition proteins. The other is the signal transfer to the lower parts of the proPO system that a microbial invader is present in hemolymph. The biochemical link between the upstream and downstream parts of the proPO system is still unknown.

In this study, we show that Tm-GRP is degraded gradually when PO activity is increased, suggesting that the 1,3-β-glucan pattern recognition protein is degraded by a serine protease(s) after recognition of non-self pathogens and after transfer of invasion signal to the downstream part of the proPO system. When we tried to identify Tm-GRP-binding protein(s) in crude hemolymph in our preliminary experiments, we found that Tm-GRP was co-localized with three other proteins (Fig. 10A). We could confirm that an aminoterminal sequence of one of these proteins perfectly matched with that of a Tenebrio masqueraider serine protease homologue (Fig. 10B), which we recently reported is involved in Tenebrio proPO activation (19). However, further biochemical studies are necessary to explain the exact details of the Tenebrio proPO activation cascade.

As shown in Fig. 4, all three insect GRPs have more than 10 Trp residues in their sequences, and their spacing and distribution are conserved. PGs from insects and humans also have a conserved distribution of Trp residues (31). Tm-GRP and other GRPs have a lectin-like property because these proteins can recognize specific microbial cell wall polysaccharides. It was reported that the conserved Trp residues of human C-type lectin are essential determinants for ligand binding and recognition of ligand (40, 50), which may also be the case for Tm-GRP. It was reported that the conserved Trp residues of human proteins can recognize specific microbial cell wall polysaccharides (31). Tm-GRP Is Degraded by Serine Protease

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Characterization and Properties of a 1,3-β-d-Glucan Pattern Recognition Protein of *Tenebrio molitor* Larvae That Is Specifically Degraded by Serine Protease during Prophenoloxidase Activation

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*J. Biol. Chem.* 2003, 278:42072-42079.  
doi: 10.1074/jbc.M307475200 originally published online August 15, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307475200

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