N-terminal GNBP homology domain of Gram-negative binding protein 3 functions as a beta-1,3-glucan binding motif in Tenebrio molitor

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The Toll signalling pathway in invertebrates is responsible for defense against Gram-positive bacteria and fungi, leading to the expression of antimicrobial peptides via NF-κB-like transcription factors. Gram-negative binding protein 3 (GNBP3) detects beta-1,3-glucan, a fungal cell wall component, and activates a three step serine protease cascade for activation of the Toll signalling pathway. Here, we showed that the recombinant N-terminal domain of Tenebrio molitor GNBP3 bound to beta-1,3-glucan, but did not activate downstream serine protease cascade in vitro. Reversely, the N-terminal domain blocked GNBP3-mediated serine protease cascade activation in vitro and also inhibited beta-1,3-glucan-mediated antimicrobial peptide induction in Tenebrio molitor larvae. These results suggest that the N-terminal GNBP homology domain of GNBP3 functions as a beta-1,3-glucan binding domain and the C-terminal domain of GNBP3 may be required for the recruitment of immediate downstream serine protease zymogen during Toll signalling pathway activation. [BMB reports 2009; 42(8): 506-510]

INTRODUCTION

Innate immunity is a crucial host defense mechanism against microbial infection in all animals. The ability of a host to distinguish between self and non-self remains a central hallmark of innate immunity (1). Pathogenic microbes possess distinct pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides in gram-negative bacteria, peptidoglycans (PGs) in gram-positive bacteria, and beta-1,3-glucans in fungi (2). Recognition of these PAMPs is achieved by a group of germ-line encoded receptors or soluble proteins via specific signal transduction pathways or via a serine protease activation cascade, respectively (3). The Drosophila Toll signalling pathway is activated upon recognition of Lys-type PGs of Gram-positive bacteria and beta-1,3-glucan of fungi, while the immune deficiency (IMD) pathway is activated primarily after recognition of diaminopimelic acid (DAP)-type PG of Gram-negative bacteria (2). Both of these pathways lead to the expression of antimicrobial peptides (AMPs) via NF-κB-like transcription factors (4-6).

In insects, bacterial lysine (Lys)-type PGs are recognized by the PG recognition protein-short form-A (PGRP-SA) and Gram-negative binding protein 1 (GNBP1) (7-10). These proteins are believed to mediate the activation of a serine protease cascade and ultimately, the cleavage of Spätzle. Cleaved Spätzle serves as a ligand for the Toll cell membrane receptor and induces the production of AMPs (6,11). However, Drosophila melanogaster GNBP3 (Dm-GNBP3), is known to be required for the detection of fungal cell wall components in the Toll signalling pathway (12). These recognition signals against bacteria and fungi are amplified in hemolymph (insect blood) by a proteolytic serine protease cascade similar to the vertebrate complement system (6). The amplification of these recognition signals represents an efficient host defense strategy in insects, which are devoid of an acquired immune system.

Recently, we analyzed the serine protease cascade that regulates the Toll pathway using the larvae of the Tenebrio molitor beetle (13, 14). This large insect enabled us to collect large amount of hemolymph, allowing us to purify several different serine proteases. Our studies demonstrated that the recognition of Lys-type PG by the PGRP-SA/GNBP1 complex activates Spätzle through the sequential activation of three different Tenebrio serine proteases: modular serine protease (MSP), Spätzle-processing enzyme-activating enzyme (SAE) and Spätzle-processing enzyme (SPE) (13). Additionally, Tenebrio GNBP3, in the presence of beta-1,3-glucan, also induces the sequential activation of a three step proteolytic cascade involving MSP, SAE and SPE zymogens, leading to the processing of pro-Spätzle into the mature Spätzle form that functions as a ligand for the Toll receptor (15). Therefore, our recent works support a model in which bacterial Lys-type PG and fungal beta-1,3-glucan recognition signals activate a common proteolytic cascade involving three different serine protease zymogens.
that are sequentially processed. This three-step proteolytic cascade leads to the maturation of Spätzle and the activation of the Toll intracellular signalling that control the synthesis of AMPs.

However, the exact biological functions of GNBP1 and GNBP3 during Toll cascade activation have yet to be determined. How the PGRP-SA/GNBP1 complex transfers Lys-type PG recognition signal to the downstream factor MSPzymogen, or which domain of GNBP3 is involved in β-1,3-glucan recognition, and how β-1,3-glucan recognition signal is transferred to MSPzymogen are not answered yet. To address these questions, we tried to express N-terminal and C-terminal domains of GNBP3 in an E. coli system, but only the recombinant N-terminal domain of GNBP3 was expressed in E. coli. Here, we revealed that N-terminal domain of GNBP3 binds to β-1,3-glucan and this domain functions as a competitive inhibitor during GNBP3-mediated Toll signalling pathway using biochemical studies.

RESULTS

N-terminal motif, but not C-terminal domain, of GNBP3 is expressed in the E. coli system

T. molitor GNBP3 (Tm-GNBP3) is 481 amino acids long with an N-terminal region of ~120 residues, a putative linker region, and a C-terminal region (~220 residues) that is similar in sequence to β-1,3-glucanases (16). The amino-terminal portion had no significant sequence similarity to any proteins other than the amino terminus of proteins that belong to the 1,3-β-D-glucan recognition protein (βGRP/GNBP family (17) (Fig. 1A). To examine the biological functions of GNBP3 during Toll signalling pathway, we tried to express two different constructs, N-terminal motif and C-terminal motif of GNBP3 with a hexa-His tag fused to the C-terminus harbouring a tobacco etch virus (TEV) protease recognition sequence in E. coli system. Isopropyl-1-thio-β-D-galactopyranoside (IPTG)-induced E. coli cells were able to express the N-terminal motif as a soluble form, but not the C-terminal motif. The recombinant N-terminal motif was affinity purified from the E. coli lysate supernatants by Ni-NTA column chromatography. After treatment with TEV protease, the generated recombinant N-terminal motif of GNBP3 was purified to homogeneity using sequential column chromatographies, such as Hitrap-Q column, Sephacryl S-200 and TSK-gel G300 columns. Purified recombinant N-terminal motif (r-N-Tm-GNBP3) was obtained in milligram quantities from 0.5-1.0 liters of bacterial cultures. SDS-PAGE analysis with Coomassie Blue staining (CBB) indicated that obtained recombinant protein solution contained a single major protein band (lanes 3 and 4 in Fig. 1B).

N-terminal GNBP homology domain of Tm-GNBP3 functions as β-1,3-glucan binding motif

It is important to determine which domain of Tm-GNBP3 is responsible for β-1,3-glucan recognition and which domain of

![Fig. 1. Isolation of recombinant N-terminal domain of Tm-GNBP3. (A) The domain structure of Tenebrio GNBP3. (B) SDS-PAGE analysis of the purified recombinant N-terminal domain of Tm-GNBP3. Pairs of lanes, such as lanes 1 and 3 or lanes 2 and 4, show the gel mobility of the purified native GNBP3 and recombinant N-terminal domain of GNBP3 (r-N-Tm-GNBP3) under reducing conditions and non-reducing conditions, respectively.](http://bfmreports.org)
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Fig. 2. In vitro characterization of r-N-Tm-GNBP3. (A) The purified r-N-Tm-GNBP3 (15 μg) was incubated and co-precipitated with insoluble β-1,3-glucan (20 mg) as follows: the purified r-N-Tm-GNBP3 (lane 1), unbound (U) supernatant fraction (lane 2) and bound (B) molecule to insoluble β-1,3-glucan fraction (lane 3) were separated on SDS-PAGE under reducing conditions and then stained with CBB. (B) In vitro reconstitution experiments for measurement of the activated Tm-SPE-mediated amidase activity were performed using the purified Tm-GNBP3, r-N-Tm-GNBP3, Tm-SAE and E2 fraction in the presence of β-1,3-glucan and Ca²⁺ as indicated. E2 fraction contains Tm-MSP and Tm-SPE zymogens as described in our recent work (13). The β-1,3-glucan-dependent amidase activities originating from the activated Tm-SPE were examined using a commercially available α-thrombin substrate (t-butyloxycarbonyl-benzyl-L-valinyl-L-prolinyl-L-arginine-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) as described previously (13). T-bars indicate mean ± SD (P < 0.05) of three independent experiments.

DISCUSSION

The detection of bacterial and fungal infections in insect innate immunity is a crucial step in the timely initiation of appropriate innate immune responses. Among pathogen recognition receptors, two GNBP proteins are essential for transferring bacterial and fungal recognition signal to the Toll signaling pathway. However, their functions of GNBP proteins were not clearly addressed because of the difficulties of purification and expression of native and recombinant GNBP proteins, respectively. Here, we expressed the N-terminal motif of GNBP3, but not C-terminal domain in E. coli and provided the biochemical evidences that r-N-Tm-GNBP3 functions as a β-1,3-glucan binding domain.

As the C-terminal domains of GNBP family molecules are known to encode the glucanase-like domains that can cleave the glucan bond of β-1,3-glucan (7-9), we speculate that

Fig. 3. Effects of r-N-Tm-GNBP3 on β-1,3-glucan-mediated antibacterial peptide inductions. Lanes indicate the antibacterial activities against S. aureus of isolated hemolymph after 36 h injection of insect saline (column 1), β-1,3-glucan (column 2, 400 ng), r-N-Tm-GNBP3 (column 3, 100 ng) and co-injection of β-1,3-glucan and r-N-Tm-GNBP3 (column 4).

Tm-GNBP3 may have two functions; one being the glucan binding ability of N-terminal domain and the other being the recognition domain for glucans. Based on the fact that the r-N-Tm-GNBP3 did not induce the proteolytic cascade activation, we can speculate that the C-terminal β-1,3-glucanase domain may be required for the recruitment and activation of the downstream serine protease. More so, r-N-Tm-GNBP3 functions as an inhibitor on GNBP3-mediated Toll signalling cas-
cascade in vitro and in vivo. These observations implicate r-N-Tm-GNBP3 may be a very valuable molecule functioning as a negative regulator for the molecular mechanism for Toll activation cascade in vivo and in vitro. Two molecules of r-N-Tm-GNBP3 than entire length Tm-GNBP3 could represent half-maximum inhibition, implying that a single molecule of Tm-GNBP3 may not recruit and activate the down-stream serine protease MSP and that GNP3 is needed to form a complex with each other for the signalling. Further studies are essential for the exact assignment of the biological functions of GNB proteins during Toll pathway.

We failed to obtain these two proteins in the supernatant fraction when we examined Tm-GNBP proteins expression in the baculovirus-insect cell expression system (data not shown). The putative reason of the difficulty of secreting insect GNBP proteins from baculovirus-infected cells could be explained by Drosophila GNBP proteins having C-terminal hydrophobic tails containing a putative glycosylphosphatidylinositol (GPI) anchor attachment site for membrane-anchoring (18), which are conserved in Tenebrio GNBP proteins. Further studies are on-going for developing the method to obtain intact GNBP proteins or GNBP domain variants by using various molecular cell biology techniques.

MATERIALS AND METHODS

Expression and purification of recombinant N- and C-terminal domains of Tm-GNBP3

Total RNA was isolated from the fat body of T. molitor larvae using ISOGEN (WAKO, Osaka, Japan). Tm-GNBP3 cDNA was prepared as previously reported (16) using SuperScript II reverse transcriptase (Life Technologies, Inc.). A DNA fragment encoding N-Tm-GNBP3 (residues 19-109) was amplified by PCR using the sense primer 5'-CCCCGAATTCCAATTTGAG-3' and the antisense primer 5'-CCTCC-TAGATCCACAAACGTCCGTCACGCA-3'. The PCR product of N-Tm-GNBP3 (19-109) was inserted into the ProEx vector using EcoR1 and Xha1 restriction sites.

The recombinant plasmid pProEX HTa-N-Tm-GNBP3 (ami-
no acid residues 19-109) was transformed into BL21 (DE3) to produce the N-terminal domain of Tm-GNBP3. Protein expression was induced by adding 0.4 mM isopropyl-D-thiogalactoside to the culture at 30°C. Cells were harvested by centrifugation 4 h after induction, and were disrupted by sonication in 60 ml of 20 mM Tris (pH 8.0) buffer containing 150 mM NaCl and 2 mM β-mercaptoethanol. The soluble lysate was centrifuged at 13,000 rpm for 30 min and the supernatant was mixed with Ni-NTA affinity resin (Qiagen, The Netherlands) that had been pre-incubated with the Tris buffer, and the mixture was stirred for 1 h at 4°C. After the slurry was loaded into the column, unbound proteins were washed with 400 ml of the Tris buffer supplemented with 20 mM imidazole. Recombinant N-Tm-GNBP3 with the hexahistidine tag was eluted with the Tris buffer supplemented with 200 mM imidazole. Eluted fractions (total 14 ml) were analyzed by SDS-PAGE.

After TEV protease treatment, the solution was purified as follows: The N-Tm-TGNBP3 was purified using Hitrap Q (1 ml, Amersham Pharmacia, USA) pre-equilibrated with 20 mM Tris (pH 8.0). The protein was eluted from the column using a 0-1 M NaCl linear gradient in 20 mM Tris buffer (pH 8.0). Fractions (10 ml) containing N-Tm-GNBP3 were pooled and concentrated to 2 ml using Centricon (Millipore, USA). Subsequently, the N-Tm-GNBP3 was purified using HiLoad Superdex 200 (Amersham Pharmacia, USA) pre-equilibrated with 20 mM Tris (pH 8.0) and containing 150 mM NaCl. Finally, the pool from HiLoad Superdex 200 was concentrated to a volume of 0.5 ml using a Centricon device (Millipore). The concentrated material was applied to a TSKgel G3000SW column (Tosoh) equilibrated in 50 mM Tris-HCl containing 3 mM EDTA (pH 6.0 containing 0.2 M NaCl). Total proteins were analyzed by SDS-PAGE on a 15% gel and stained with Coomassie blue. The purity of the protein sample was estimated through the protein band intensities on the Coomassie-blue-stained gel. Also, the N-terminal amino acid sequence of purified r-N-Tm-GNBP3 was carried out to verify the identities of the purified proteins. The purified protein was stored at 4°C for use within a week, or frozen at −80°C until use.

Measurement of the β-1.3-glucan-specific amidase activity

To evaluate the amidase activity in the sample, commercially available α-thrombin substrate (t-butyloxy-carbonyl-benzyl-L-valinyl-L-prolinyl-L-arginine-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) was used. This α-thrombin substrate is specifically cleaved by the active form of Trm-SPE (13). The substrate was dissolved in dimethylformamide according to the manufacturer’s instructions. A 10 μl sample of crude hemolymph or a fraction from column chromatography was incubated with β-1.3-glucan in the presence of 490 μl reaction solution, which contains 20 μM substrate in 20 mM Tris-HCl buffer (pH 8.0). After incubating the mixture at 30°C for 1 h, 500 μl of 17% (v/v) acetic acid was added to terminate enzyme reaction. Specific amidase activity can be detected by a fluorescence spectrophotometer at λem=380 nm and λex=460 nm. One unit of the amidase activity was defined as the amount that liberated 1 nmol of 7-amino-4-methylcoumarin per min.

Assay of antibacterial activity

Antibacterial susceptibility disc tests against S. aureus were performed as previously described (13), using lyophilized hemolymph from 100 μl of a whole extract resulting from the injection of insect saline, β-1,3-glucan (400 ng), r-N-Tm-GNBP3 (100 ng) and co-injection of β-1,3-glucan (400 ng) and r-N-Tm-GNBP3 (100 ng). Briefly, the bactericidal activity of
the hemolymph was assayed against *S. aureus* (strain Cowan 1) as an indicator bacterium. These bacteria were harvested in the exponential phase of growth, and then suspended in 10 mM sodium phosphate buffer containing 130 mM NaCl, pH 6.0. Antibacterial susceptibility disc tests against *S. aureus* were performed as previously described (13), using lyophilized hemolymph from 100 μl of a whole extract harvested at 36 h after injection. The zones of inhibition were estimated after overnight incubation.

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**REFERENCES**


