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Gram-negative Bacteria-binding Protein, a Pattern Recognition Receptor for Lipopolysaccharide and β-1,3-Glucan That Mediates the Signaling for the Induction of Innate Immune Genes in *Drosophila melanogaster* Cells*

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Pattern recognition receptors, non-clonal immune proteins recognizing common microbial components, are critical for non-self recognition and the subsequent induction of Rel/NF-κB-controlled innate immune genes. However, the molecular identities of such receptors are still obscure. Here, we present data showing that *Drosophila* possesses at least three cDNAs encoding members of the Gram-negative bacteria-binding protein (DGNBP) family, one of which, DGNBP-1, has been characterized. Western blot, flow cytometric, and confocal laser microscopic analyses demonstrate that DGNBP-1 exists in both a soluble and a glycosylphosphatidylinositol-anchored membrane form in culture medium supernatant and on *Drosophila* immunocompetent cells, respectively. DGNBP-1 has a high affinity to microbial immune elicitors such as lipopolysaccharide (LPS) and β-1,3-glucan whereas no binding affinity is detected with peptidoglycan, β-1,4-glucan, or chitin. Importantly, the overexpression of DGNBP-1 in *Drosophila* immunocompetent cells enhances LPS- and β-1,3-glucan-induced innate immune gene (NF-κB-dependent antimicrobial peptide gene) expression, which can be specifically blocked by pretreatment with anti-DGNBP-1 antibody. These results suggest that DGNBP-1 functions as a pattern recognition receptor for LPS from Gram-negative bacteria and β-1,3-glucan from fungi and plays an important role in non-self recognition and the subsequent immune signal transmission for the induction of antimicrobial peptide genes in the *Drosophila* innate immune system.

The innate immunity in *Drosophila* is an efficient host defense system aimed at preventing microbial infections (1–3). Upon microbial infection, insects rapidly recognize an invading pathogen as non-self and synthesize a battery of innate immune genes such as antimicrobial peptides (1–3). The induction of antimicrobial peptide genes is regulated by Rel/NF-κB factors in either Toll-dependent and/or -independent Rel/NF-κB signaling pathways (4–7). In both signaling pathways, the activation of Rel/NF-κB factors is regulated by the *Drosophila* homolog of the mammalian IκB kinase (8). In mammals, pathogen-induced innate immune signaling pathways are also achieved through Toll → IκB kinase → Rel/NF-κB factors (9–14).

Although striking similarities have been observed between the intracellular innate immune signaling pathways in insects and mammals (1, 4, 8, 9, 15, 16), the recognition process for non-self remains a challenging field in innate immune signal transduction. It has been hypothesized that the innate immune system can detect invading pathogens by virtue of "non-clonal pattern recognition receptors" that interact with common microbial structures and deliver an immune signal to the host cells (16, 17). In humans, distinct membrane Toll-like receptors can directly bind common bacterial components such as LPS,[1] bacterial lipoprotein, and peptidoglycan and subsequently initiate an intracellular Rel/NF-κB signaling pathway leading to innate immune gene induction (9–13). In *Drosophila*, although Toll and the related molecule 18-Wheeler are involved in the induction of antimicrobial peptide genes (4, 18), *Drosophila* Toll does not function as a pattern recognition receptor (19). Instead of microbial cell wall components, an active form of the spaetzle gene product generated by the proteolytic cascade is thought to be the extracellular ligand for Toll in the immune response (4, 19).

Recently several soluble recognition molecules including Gram-negative bacteria-binding protein (20–22), peptidoglycan binding protein (23, 24), LPS-and β-1,3-glucan binding protein (25, 26), and β-1,3-glucan recognition protein (27, 28) have been found in various invertebrates and are proposed as pattern recognition receptors. The key question, however, that remains is whether those soluble pattern recognition molecules

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF228472 (DGNBP-1), AF228473 (DGNBP-2), and AF228474 (DGNBP-3).

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[1] The abbreviations used are: LPS, lipopolysaccharide; GNBP, Gram-negative bacteria-binding protein; PCR, polymerase chain reaction; LGBP, LPS-and β-1,3-glucan binding protein; CCF-1, coelomic cytolytic factor-1; βGRP, β-1,3-glucan recognition protein; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-PCR; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

can truly mediate the induction of innate immune genes in response to microbial infection or to the presence of microbial cell wall components. In this study, we have addressed two questions. First, do the membrane and/or soluble forms of pattern recognition receptors exist in *Drosophila* immune cells? And second, if so, do they transmit immune signaling across the membrane for the induction of innate immune genes? To address these questions, we have cloned three novel *Drosophila* Gram-negative bacteria-binding proteins (DGNBPs) and showed that DGNBP-1 (i) exists in both soluble and membrane-bound forms, (ii) is a pattern recognition protein for specific microbial components, and (iii) mediates induction of various κB-dependent innate immune genes in response to microbial challenges.

EXPERIMENTAL PROCEDURES

Insect Cell Culture—*Drosophila* immunocompetent Schneider cells (ATCC CRL-1963) and *l(2)mbn* cells were maintained exactly as described previously (29). Stably transformed cells expressing DGNBP-1 were maintained in the presence of 300 μg/ml hygromycin.

cDNA Cloning of DGNBP Family—Except when specially mentioned, all DNA and RNA manipulations were carried out using standard techniques (30). With a synthetic primer pair (sense: 5′-ATG CCA GGA TTG TGC ATT G-3′; antisense: 5′-GTC CAA AGG TAT AGA ACA TC-3′) derived from a *Drosophila* expressed sequence tag (EST) clone (LD 15841) showing homology to the amino acid sequence of *Bombyx* GNBP (20), we amplified and isolated a specific 300-base pair polymerase chain reaction (PCR) product from a *Drosophila l(2)mbn* cDNA library (kindly provided from Dr. D. Hultmark, University of Umeå, Sweden). The PCR fragment was used as a probe to screen a λZap II *l(2)mbn* cDNA library. Partial sequences of two other DGNBPs (DGNBP-2 (GH 07433) and DGNBP-3 (LP 05991)), deposited by the EST project (Berkeley *Drosophila* Genome Project), were identified by BLAST algorithm-based GenBank™ search using the DGNBP-1 full sequence. Full cDNA sequences of DGNBPs were determined using an A.L.F. express automatic sequencer (Amersham Pharmacia Biotech).

RT-PCR Analysis—RT-PCR was performed exactly as described previously (8). The sequences of the specific primers for each of the GNBPs are the following: DGNBP-1, sense, 5′-CAC ACC GAC TGT GGA GCT CCT TG-3′ and antisense, 5′-GGC TGC GCC AGA TCT TGA TAC-3′; DGNBP-2, sense, 5′-ATG AGG TGG GAA TTT CTG C-3′ and antisense, 5′-TCA CCC TGG TTT CAC TCT T-3′; DGNBP-3, sense, 5′-AAG GCT AAG ATC GAT GTT-3′ and antisense, 5′-CGT CTT CGC GAT AAC CCA GTC-3′. We used an antibacterial cecropin gene as a marker for immune-inducible genes and the constitutive β-actin gene for an internal control.

Bacterial Expression and Polyclonal Antibody Production of the DGNBP-1—DGNBP-1 cDNA was digested with *Sac*I and *Pst*I, which gave a 1.3-kilobase fragment (nucleotides 275–1582), which was used to produce histidine-tagged recombinant protein according to the manufacturer's instructions (Qiagen). Rabbit polyclonal antibody using recombinant protein was produced, and specific anti-DGNBP-1 antibody was purified by affinity as previously described (31).

Flow Cytometric Analysis and Confocal Laser Microscopy—For the detection of the membrane-bound form of endogenous DGNBP-1, immunocompetent Schneider cells (2×10^6) were washed three times with ice-cold phosphate-buffered saline (PBS) and incubated in PBS containing 2% fetal bovine serum for 10 min at 4 °C. The rabbit anti-DGNBP-1 antiserum was used at 1:100 dilution in PBS for 30 min at 4 °C. Secondary fluorescein isothiocyanate-conjugated goat anti-rabbit antibody was used at 1:100 dilution in PBS for 1 h at 4 °C. After each antibody incubation, cells were washed three times for 10 min with PBS at 4 °C. After final washing, cells were fixed with 4% paraformaldehyde in PBS. Surface-stained cells were analyzed by confocal microscopy (Leica) and FACScan™ flow cytometer supported by Lysis II software (Becton Dickinson).

Overexpression of DGNBP-1 in Drosophila Cells—The DGNBP-1 open reading frame was subcloned into pMT/V5 vector (pMT/V5-DGNBP-1) under the control of the metallothionein promoter (Invitrogen). Cells stably expressing DGNBP-1 were generated as described previously (8). Expression was induced in pools of cells by addition of $CuSO_4$ to the culture medium at a final concentration of 500 μM as described previously (8). Cells were induced for 36 h before use. The DGNBP-1-ΔC construct, a deletion mutant lacking the last 10 amino acids in the COOH-terminal hydrophobic tail was also constructed and used for the generation of a stable cell line.

Phosphatidylinositol-specific Phospholipase C (PI-PLC) Treatment of DGNBP-1-overexpressed Cells—PI-PLC treatment was carried out essentially as described previously (32). Briefly, DGNBP-1-overexpressed cells (10^6) were washed three times with ice-cold PBS and incubated at 30 °C for 1 h in 50 μl of PBS with or without 1 unit of PI-PLC (Sigma). According to manufacturer's information, one unit will liberate one unit of acetylcholinesterase per min from a membrane-bound crude preparation at pH 7.4 at 30 °C for 10 min. After brief centrifugation, an aliquot (20 μl) of supernatant was subjected to Western blot analysis using affinity-purified anti-DGNBP-1 antibody.

Northern Blot Analysis—Cells were stimulated with either LPS (10 μg/ml) or β-1,3-glucan (10 μg/ml) for 3 h. Total RNA extraction and Northern blot experiments were carried out as described previously (20). The open reading frame regions of the cDNAs encoding cecropin A1 (33), drosomycin (34), and attacin (35) were amplified by PCR, and each amplified PCR product was used as probe. The β-actin cDNA was used as an internal standard probe (36). For inhibition experiment by anti-DGNBP-1 antiserum, cells were pretreated with either DGNBP-1 antiserum or pre-immune serum at the final concentration of 1% for 1 h at room temperature before stimulation.

Binding Assay—Curdlan (β-1,3-glucan), peptidoglycan (β-1,4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine), chitin (β-1,4-*N*-acetyl-D-glucosamine) and cellulose (β-1,4-glucan) were used for the *in vitro* binding assay of DGNBP-1. One hundred μg of each insoluble polysaccharide was added in 300 μl of the supernatant of DGNBP-1 overexpressed stable cell line or in 5 μg of purified DGNBP-1 and incubated at room temperature with mild agitation for 1 h. The mixture was centrifuged ($10{,}000 \times g$ for 2 min) and the pellet was washed three times with 1 ml of washing buffer (10 mM Tris, pH 7.5, 500 mM NaCl, 0.02% Tween 20). The proteins bound on insoluble polysaccharide were detached by adding SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blot analysis using affinity-purified anti-DGNBP-1 antibody. The LPS binding assay was carried out by essentially the same method as described above except the binding mixture was centrifuged at $20{,}000 \times g$ for 10 min at each step to precipitate small particles of LPS.

RESULTS

Structural Features and Expression Patterns of the DGNBP Family—To investigate the functional pattern recognition receptor(s) in *Drosophila* immune system, we first BLAST-searched the *Drosophila* database (BDGF *Drosophila* Genome Project, Berkeley, CA) using the NH_2-terminal amino acid sequence of Gram-negative bacteria-binding protein, a candidate immune recognition protein originally identified in *Bombyx mori* (20). One positive clone (DGNBP-1) was isolated from a *Drosophila l(2)mbn* cell library as described under "Experimental Procedures." Sequencing analysis showed that DGNBP-1 contains an open reading frame of 1482 nucleotides corresponding to 494 amino acids. Using an amino acid sequence deduced from the full DGNBP-1 cDNA, we identified two highly homologous partial sequences deposited in the *Drosophila* EST database. The entire nucleotide sequences of these two DGNBP homologs (DGNBP-2 and-3) were also determined. The sequence alignment of the three newly cloned DGNBPs showed that DGNBP-1 shares 30 and 26% identity with DGNBP-2 and DGNBP-3, respectively (Fig. 1*A*).

Furthermore, to facilitate future genetic analysis of these DGNBPs, chromosomal localizations of DGNBPs were established from the *Drosophila* Genome Database based on the known genes situated in the proximity of DGNBPs. DGNBP-1 and -2 were co-located at 74D-75C1 between *ftz-f1* (74DS4-5) and *term* (75C1-2). DGNBP-3 was found at 66E-F between *dally* (66E1) and *argk* (66F1).

Interestingly, all DGNBPs contain a β-1,3-glucanase-like domain homologous to *Bacillus circulans* β-1,3-glucanase (37) (Fig. 1*B*). The two glutamic acid residues (Fig. 1*B, asterisks*) correspond to active site residues in the glucanase domain of *B. circulans* β-1,3-glucanase. Like other insect β-1,3-glucanase-like domain-containing recognition proteins (20, 27, 28), these

FIG. 1. **Structural feature of DGNBPs.** *A,* multiple alignment of DGNBPs. Identical amino acid residues are *boxed*. The numbers to the right indicate amino acid position of the encoded protein. *B*, multiple alignment of the β-1,3-glucanase homology domain of glucanase motif-containing proteins. The numbers to the *right* indicate the initial amino acid position to the last amino acid position of the glucanase homology stretch of each protein. Glutamic acid residues corresponding to the active site of the *B. circulans* β-1,3-glucanase A1 are marked with *asterisks*. *Ag-GNBP*, *A. gambiae* GNBP (AJ001042); *Bci-beta-gluc*, *B. circulans* β-1,3-glucanase A1 (P23903); *Bm-beta-GRP*, *B. mori* β-1,3-glucan recognition protein (AB026441); *Bm-GNBP*, *B. mori* GNBP (L38591); *CCF-1*, *E. foetida* coelomic cytolytic factor (AF030028); *DGNBP*, *D. melanogaster* GNBP; *Factor G*, *T. tridentatus* β-1,3-glucan-sensitive coagulation factor G α-chain (D16622); *LGBP*, *P. leniusculus* LPS-and β-1,3-glucan-binding protein (AJ250128); *Ms-GRP*, *M. sexta* β-1,3-glucan recognition protein (AF177982). The alignment is optimized by introducing gaps (−) using the Clustal W 1.7 program.

residues are altered in the DGNBPs, suggesting that DGNBPs have lost glucanase activity. A sequence comparison of the β-1,3-glucanase homology domain of DGNBPs (Fig. 1*B*) with known β-1,3-glucanase domain-containing recognition protein family shows that DGNBP-1 is most homologous to *B. mori* GNBP (20) with 44% identity followed by DGNBP-2 with 40% identity, *Manduca sexta* β-1,3-glucan recognition protein (βGRP) (28) and *B. mori* βGRP (27) with 39.5% identity, DGNBP-3 with 39% identity, *Anopheles gambiae* GNBP (21) with 33% identity, LPS- and β-1,3-glucan binding protein (LGBP) of *Pacifastacus leniusculus* (26) with 32% identity, *B. circulans* β-1,3-glucanase (37) with 32% identity, coelomic cytolytic factor-1 (CCF-1) of *Eisenia foetida* (25) with 29% identity, and *Tachypleus tridentatus* β-1,3-glucan-sensitive coagulation factor G α-chain (38) with 23% identity.

To determine the transcriptional regulation of DGNBPs during *Drosophila* development, we performed RT-PCR analysis using total RNA isolated from different developmental stages utilizing specific primer pairs for each DGNBP. The levels of mRNA encoding each DGNBP were normalized in relation to the levels of control RNA encoding β-actin. The expression of the *DGNBP-1* gene was detected throughout all *Drosophila* life stages from egg to imago (Fig. 2*A*). However, the mRNA of DGNBP-2 and -3 showed very weak signals during embryonic development (Fig. 2*A*).

We next examined the induction profile of DGNBP mRNA following infection. A RT-PCR analysis for DGNBPs was performed using *Drosophila* adults and a *Drosophila* immunocompetent Schneider and *l(2)mbn* cell line following a bacterial challenge and fungal infection. We also performed RT-PCR for a constitutively expressed β-actin gene and an inducible anti-

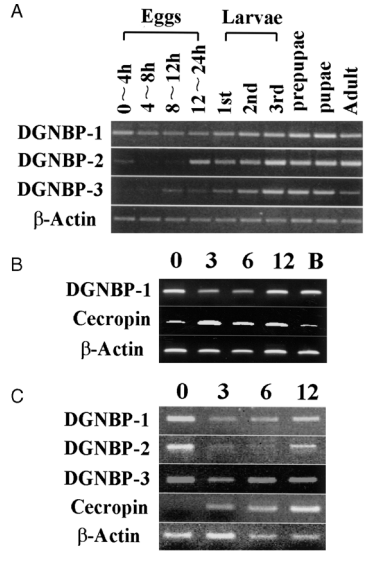

FIG. 2. **Expression profiles of DGNBPs.** *A*, RT-PCR analysis of DGNBPs at different *Drosophila* developmental stages. Total RNA from *Drosophila* at several developmental stages was extracted and used for RT-PCR analysis. *B*, RT-PCR analysis of DGNBP-1 in immune-challenged *Drosophila* adults. *Drosophila* male adults were unchallenged (*lane 0*), *Escherichia coli*-challenged for 3, 6, and 12 h (*lanes 3*, *6,* and *12*, respectively) and *Beauveria bassiana*-challenged for 36 h (*lane B*) as described by Lemaitre *et al.* (42), and used for RT-PCR analysis. *C*, RT-PCR analysis of DGNBPs in immunocompetent *Drosophila* Schneider cells. Cells were treated with LPS (10 μg/ml) for 0, 3, 6, and 12 h.

bacterial cecropin A gene. No additional up-regulation was observed following a microbial challenge, which demonstrated that endogenous DGNBPs are constitutively transcribed in *Drosophila* adults and immunocompetent Schneider cells (Fig. 2, *B* and *C*). Similar expression profiles were obtained when we performed RT-PCR using *l(2)mbn* cells (data not shown). Under the same conditions, the cecropin A gene is markedly induced. Interestingly, we observed a significant down-regulation of DGNBPs during the early phase (3–6 h) of bacterial infection (Fig. 2, *B* and *C*).

DGNBP-1 Exists in Both a Soluble and Membrane-bound Glycosylphosphatidylinositol (GPI)-anchored Form in Drosophila Cells, and the COOH-terminal Hydrophobic Tail Is Necessary for Membrane Localization—DGNBPs have COOH-terminal hydrophobic tails containing a putative GPI anchor attachment site, which suggests the existence of a membrane-bound form. As membrane localization of the recognition molecules is necessary for signal transmission across the membrane of immune cells, we examined the cellular localization of DGNBP-1 in immunocompetent Schneider cells. We first generated a polyclonal antibody using bacterial-expressed DGNBP-1. Western blot analysis showed that this antiserum specifically recognized an endogenous polypeptide with an apparent molecular mass of 55 kDa in Schneider cells whereas the pre-immune serum derived from the same rabbit did not recognize this polypeptide (Fig. 3*A*). This antibody specifically recognized recombinant DGNBP-1 but not recombinant DGNBP-2 or -3 (data not shown). With this specific anti-DGNBP-1 antiserum, we used cell surface staining methods in a non-permeable condition. Flow cytometric analysis and confocal laser microscopy showed that the endogenous DGNBP-1 is located on the surface of the immunocompetent Schneider cells (Fig. 3, *B–E*).

We next examined whether the COOH-terminal hydrophobic tail is necessary for membrane localization of DGNBP-1. For this purpose, we generated a cell line stably expressing a DGNBP-1-ΔC mutant form lacking the COOH-terminal hydrophobic tail (deletion of the last 10 amino acids). Overexpression of the DGNBP-1-ΔC mutant was initiated by adding $CuSO_4$, and a membrane fraction was prepared. Western blot analysis showed that this DGNBP-1 mutant form was not detected in the cell membrane fraction whereas the DGNBP-1 wild type was detected in the membrane fraction of the $CuSO_4$-induced cells (Fig. 3*F*). This result indicates that the COOH-terminal hydrophobic tail is necessary for the normal membrane localization of the DGNBP-1 protein.

To discern whether DGNBP-1 is GPI-anchored, we treated DGNBP-1-overexpressed cells with PI-PLC. The proteins released from cells were subjected to Western blot analysis. The results indicate that DGNBP-1 can only be detected in the supernatant of cells treated with PI-PLC, demonstrating that DGNBP-1 is a GPI-anchored membrane protein (Fig. 3*G*).

In our previous study, *Bombyx* GNBP was purified as a soluble form from the immunized hemolymph (20). It is possible that DGNBP-1 exists both as soluble and a GPI-anchored membrane-bound form. To establish whether DGNBP-1 can also exist as a soluble form, we generated a stable cell line under the control of a metallothionein promoter producing a wild-type GNBP-1 because of the small amount of soluble DGNBP-1 produced in the medium of cultured cells. In the copper-induced condition, we also detected a large amount of overexpressed DGNBP-1 in the culture supernatant (Fig. 3*H*). These results show that DGNBP-1 exists both as a soluble and a GPI-anchored membrane-bound form in cultured *Drosophila* immune cells.

DGNBP-1 Can Recognize the Pattern Motif of β-1,3-Glucan and LPS—In the *Drosophila* innate immune system, as in the human innate immune system, the pattern motifs of the microbial cell wall components (such as LPS from Gram-negative bacteria, peptidoglycan from Gram-positive bacteria, and β-1,3-glucan from yeast) can initiate innate immune signaling. To examine the binding specificity of DGNBP-1 with these immune elicitors, we incubated various insoluble oligosaccharide polymers with DGNBP-1. Subsequent to washing as described under "Experimental Procedures," the proteins bound to the precipitates were extracted with SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blot using affinity-purified anti-DGNBP-1 antibody. The results show that DGNBP-1 was specifically detected in the extract

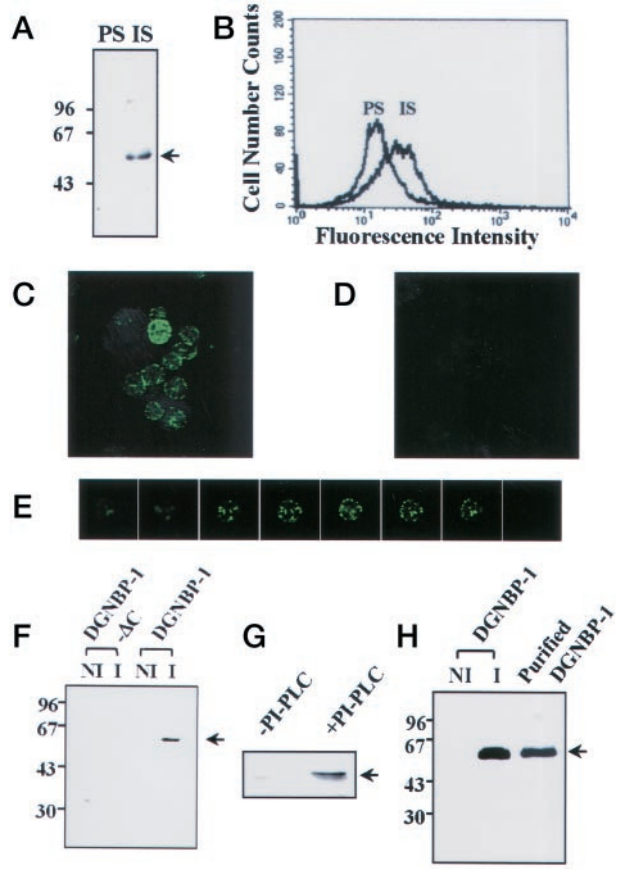

FIG. 3. **Localization of DGNBP-1.** Normal untransfected Schneider (*A–E*) cells and DGNBP-1-overexpressed stable cell lines (*F–H*) were used for the experiments. *A*, Western blot of Schneider cell lysates using anti-DGNBP-1 antiserum (*IS*) or pre-immune serum (*PS*). The molecular mass markers are indicated in kDa. *B*, flow cytometric analysis of immunocompetent Schneider cells stained by either pre-immune serum (*PS*) or anti-DGNBP-1 antiserum (*IS*). *C*, immunofluorescence detection of membrane-bound DGNBP-1 in immunocompetent Schneider cells by anti-GNBP-1 antiserum. *D*, control immunofluorescence detection by pre-immune serum. *E*, serial section of DGNBP-1-stained cell. Eight serial sections were obtained by confocal image analysis program. *F*, Western blot analysis of membrane fraction of Schneider cells stably expressing either DGNBP-1 wild type (*DGNBP-1*) or DGNBP mutant form lacking COOH-terminal hydrophobic tail (*DGNBP-1-ΔC*). Cells were treated with or without $CuSO_4$ for 36 h, sonicated for 15 s, and centrifuged at 20,000 × *g* for 10 min. The pellets containing membrane fraction were washed and extracted with 8 M urea. Aliquots of membrane extracts were analyzed by Western blot analysis. *G*, digestion of membrane-bound form of DGNBP-1 by PI-PLC. Cells stably expressing DGNBP-1 were washed three times with PBS and incubated with or without PI-PLC for 1 h at 30 °C. After centrifugation, supernatants were subjected to Western blot analysis. *H*, detection of the soluble form of DGNBP-1 in culture media. Cells (DGNBP-1) were incubated with or without $CuSO_4$ for 36 h. Supernatant (300 μl) was concentrated by trichloroacetic acid precipitation and subjected to Western blot analysis.

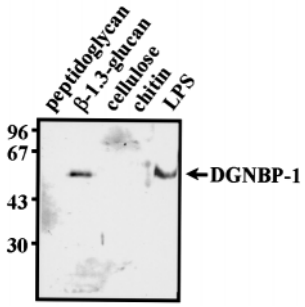

FIG. 4. **Binding activity of DGNBP-1.** *In vitro* binding assay was performed as described under "Experimental Procedures" using various microbial immune elicitors (peptidoglycan, β-1,3-glucan, and LPS) and related polysaccharide structures (cellulose and chitin). The molecular mass markers are indicated in kDa.

from the binding assay with β-1,3-glucan and LPS, whereas no binding activity was observed with peptidoglycan, β-1,4-glucan, or chitin (Fig. 4). Thus, the binding of DGNBP-1 to β-1,3-glucan and LPS seems to be specific.

Involvement of DGNBP-1 in the Induction of Innate Immune Genes—Given that DGNBP-1 can recognize LPS and β-1,3-glucan *in vitro* and that it is located on the membrane of immunocompetent cells, the possible involvement of DGNBP-1 in the induction of *Drosophila* Rel/NF-κB-controlled innate immune genes was investigated. For this purpose, we first generated a cell line stably expressing DGNBP-1 under the control of a metallothionein promoter and used this to analyze immune gene regulation. Following β-1,3-glucan or LPS stimulation, we used a Northern blot analysis with specific probes for well known κB-dependent antimicrobial peptide genes (drosomycin, cecropin, and attacin). The results demonstrate that when the DGNBP-1 is overexpressed, the immune inducibility of all examined antimicrobial peptide genes was greatly enhanced by 2–4 times over that of control cells (Fig. 5, *A* and *B*). Similar results were obtained when we examined the time course activation of antimicrobial gene expression (Fig. 5*C*). In our previous report (8) and also in the control experiments, $CuSO_4$ treatment in the untransfected cells or cell line stably expressing an unrelated *Drosophila* protein had no noticeable effect on the immune inducibility of antimicrobial genes (data not shown). These results show that DGNBP-1-overexpressed cells are more responsive to LPS and β-1,3-glucan for the induction of innate immune genes, thereby indicating the involvement of DGNBP-1 in the LPS or β-1,3-glucan signal transduction pathway at least when DGNBP-1 is overexpressed in *Drosophila* immune cells.

To see whether endogenous DGNBP-1 is indeed a pattern recognition receptor for the transmission of NF-κB signaling across the membrane, we pretreated immunocompetent Schneider cells with monospecific DGNBP-1 antiserum to inhibit the binding capacity of endogenous DGNBP-1 prior to LPS stimulation. Northern blot analysis was performed to measure antimicrobial peptide gene expression in a DGNBP-1-antiserum-treated condition and control pre-immune serum-treated condition. The results show that the LPS-inducibility of attacin, cecropin, and drosomycin was greatly impaired by pretreatment with DGNBP-1 antiserum but not by pre-immune control serum (Fig. 6). This suggests that endogenous DGNBP-1 is an essential signal transducer for inducibility of innate immune genes in cultured *Drosophila* immune cells.

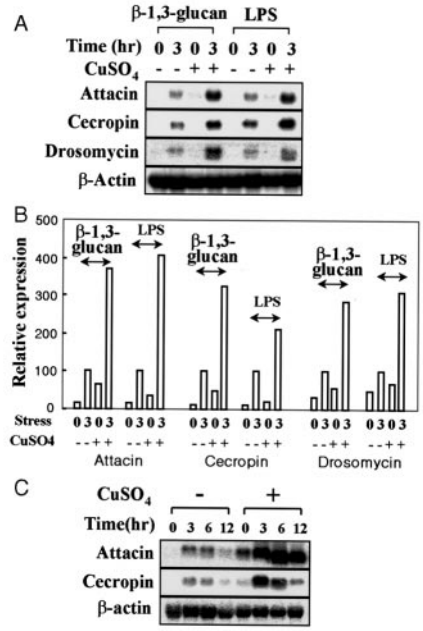

FIG. 5. **Enhanced inducibility of *Drosophila* innate immune genes following β-1,3-glucan or LPS treatment in DGNBP-1-overexpressed cells.** *A*, Northern blot analysis of innate immune gene expression. Cells stably expressing DGNBP-1 under control of metallothionein promoter were treated with or without $CuSO_4$ (500 μM) for 36 h. Cells were then incubated with β-1,3-glucan (10 μg/ml) or LPS (10 μg/ml) for 3 h. The expression level of various innate immune genes was measured by performing Northern blot analysis. *B*, quantification of innate immune gene expression. The signals obtained from Northern blot analysis were quantified by PhosphorImager (Fuji). Signals for each antimicrobial peptide gene was normalized with the corresponding value of β-actin signals. In each antimicrobial peptide gene, the expression level following β-1,3-glucan or LPS treatment in the absence of DGNBP-1 overexpression was taken arbitrarily as 100, and the results are presented as relative expression. Each *bar* represents the average of four independent experiments. *C*, time course activation of cecropin and attacin genes in DGNBP-1-overexpressed cells following LPS stimulation. Cells stably expressing DGNBP-1 under control of metallothionein promoter were used in this study. Cells were incubated in the absence or presence of $CuSO_4$ for 36 h and then incubated with or without LPS (10 μg/ml) for 0, 3, 6, and 12 h. Northern blot analysis was performed as described under "Experimental Procedures." Data are representative from three independent experiments.

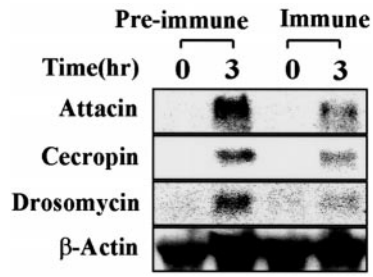

FIG. 6. **LPS-induced up-regulation of innate immune genes is inhibited by blocking of endogenous DGNBP-1.** *Drosophila* immunocompetent Schneider cells were treated with either monospecific DGNBP-1 antiserum or control pre-immune serum for 1 h at room temperature prior to LPS stimulation (10 μg/ml for 3 h) Northern blot analysis was performed to measure antimicrobial peptide gene expression. Data are representative from three independent experiments.

DISCUSSION

The initiation of the innate immune system is an important means of host defense in all eukaryotes (1, 39). Analysis of the regulation of innate immune genes such as antimicrobial peptide genes in *Drosophila* have been particularly fruitful and is providing new directions for the analysis of the mammalian host defense system (1, 39). It is now clear that the Toll receptor family, originally identified in *Drosophila* dorsoventral development, mediates the innate immune response through Rel/NF-κB in insects and mammals (4, 7, 9–14, 18). Although increasing amounts of information on the intracellular signal-

ing pathway leading to antimicrobial peptide gene induction have been documented in *Drosophila* (1–8), no pattern recognition protein directly involved in this signal transduction has been reported. Because the recognition of microbial cell wall components is an essential initial step for intracellular innate immune signaling, we focused on the early recognition event of the innate immune system.

In the present study, we have shown that DGNBP-1 specifically recognizes common immune elicitors, such as LPS and β-1,3-glucan. However, similar sugar motifs such as chitin and cellulose are not efficient ligands for DGNBP-1. Thus, the binding specificity of DGNBP-1 seems to be restricted to a common structural motif between LPS and β-1,3-glucan. Previously, we observed that *Bombyx* GNBP exclusively binds Gram-negative bacteria (20). In the case of *Anopheles* GNBP, the binding specificity is unknown, but the induction of *Anopheles* GNBP mRNA is more responsive to Gram-positive bacteria than to Gram-negative bacteria, and yeast is ineffective as an inducer (21). Interestingly, *Anopheles* GNBP is also up-regulated by the malaria parasite (22). These results suggest that different members of the GNBP family may have different recognition specificities for the recognition of diverse pathogens. It is also possible that DGNBP-2 and DGNBP-3 have different pattern recognition characteristics than DGNBP-1 and may serve in the recognition of different microbial pathogens. Very recently, Ochiai and Ashida (27) proposed the existence of more than two kinds of domains together with the 100 amino acids of the NH_2-terminal region that are implicated in β-1,3-glucan recognition of β-1,3-glucanase domain-containing recognition proteins. Thus, the binding domain of β-1,3-glucanase domain-containing recognition proteins seems to be more complex than previously thought. More extensive binding studies of the entire β-1,3-glucanase domain-containing recognition protein family will elucidate this issue. For instance, the binding characteristics of DGNBP-1 are similar to recently cloned β-1,3-glucanase domain-containing soluble proteins such as LGBP, CCF-1, and βGRPs from different species of invertebrates (25–28). These proteins are known to be involved in the prophenoloxidase activation system, a constitutive immune cascade found in body fluid (25–28). However, unlike these β-1,3-glucanase domain-containing recognition proteins, DGNBP-1 exists as both a soluble and a GPI-anchored membrane-bound form in cultured *Drosophila* immune cells. As the COOH-terminal is shown to be important for the membrane attachment of DGNBP-1, soluble DGNBP-1 is probably generated by a post-translation modification.

Pattern recognition receptors are required to possess at least two sequential functional capacities for the correct initiation of the innate immune system: (i) evaluate non-self pathogens by pattern recognition capacities, and (ii) deliver a danger signal to immune cells for *de novo* synthesis of innate immune molecules. No such pattern recognition receptor has been described in *Drosophila* or any other insect. We have shown that overexpression of both forms of DGNBP-1 greatly enhances the immune inducibility of antimicrobial peptide genes in response to LPS and β-1,3-glucan. Furthermore, blocking endogenous DGNBP-1 by the DGNBP-1 antibody inhibited the LPS-induced inducibility of antimicrobial peptide genes. These results correlate well with our binding studies and suggest that DGNBP-1 is a functional pattern recognition receptor for LPS and β-1,3-glucan, which plays the role of an immune-signaling mediator across the cell membrane. However, at present we cannot explain how the soluble and membrane-bound form of DGNBP-1, lacking a cytoplasmic signaling domain, intervenes in immune signal transmission for the induction of antimicrobial peptide genes. A similar situation was observed in the mammalian GPI-anchored CD14, a well known pattern recognition molecule for LPS also lacking a cytoplasmic domain (40). In the case of CD14, it serves as a co-receptor for the Toll-like receptor in response to microbial infection (11, 41). The Toll-like receptor contains a cytoplasmic domain homologous to the type-I interleukin-1 receptor, which is essential for innate immune signal transduction. Whether DGNBP-1 also synergistically cooperates with other proteins containing a cytoplasmic domain (*e.g. Drosophila* Toll/18-Wheeler or with other unknown receptors) for signal transmission remains to be determined.

In conclusion, our results suggest that DGNBP-1 is a functional pattern recognition receptor for LPS and β-1,3-glucan and mediates innate immune signaling for the induction of antimicrobial peptide gene induction in cultured *Drosophila* immune cells. To our knowledge, this is the first report of an invertebrate pattern recognition protein directly involved in both recognition and transmission of intracellular immune signaling. More detailed *in vivo* genetic studies will allow us to better understand the role(s) of pattern recognition receptors in the innate immune system in *Drosophila* and perhaps in humans.

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REFERENCES

1. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., Jr., and Ezekowitz, R. A.(1999) *Science* **284,** 1313–1318
2. Hultmark, D. (1993) *Trends Genet.* **9,** 178–183
3. Engström, Y. (1998) in *Molecular Mechanisms of Immune Response in Insects* (Brey, P. T., and Hultmark, D., eds) pp. 211–244, Chapman and Hall Ltd., London
4. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.-M., and Hoffmann J. A. (1996) *Cell* **86,** 973–983
5. Wu, L. P., and Anderson, K. V. (1998) *Nature* **392,** 93–97
6. Meng, X., Khanuja, B. S., and Ip, Y. T. (1999) *Genes Dev.* **13,** 792–797
7. Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.-M., and Hoffmann, J. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9465–9469
8. Kim, Y.-S., Han, S.-J., Ryu, J.-H., Choi, K.-H., Hong Y.-S., Chung, Y.-H., Perrot, S., Raibaud, A., Brey, P.-T., and Lee, W.-J. (2000) *J. Biol. Chem.* **275,** 2071–2079
9. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997) *Nature* **388,** 394–397
10. Janeway, C. A., Jr., and Medzhitov, R. (1999) *Curr. Biol.* **9,** 879–882
11. Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J., and Modlin, R. L. (1999) *Science* **285,** 732–736
12. Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999) *J. Biol. Chem.* **274,** 17406–17409
13. Cario, E., Rosenberg, I. M., Brandwein, S. L., Beck, P. L., Reinecker, H. C., and Podolsky, D. K. (2000) *J. Immunol.* **164,** 966–972
14. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 588–593
15. O'Neill, L. A., and Greene, C. (1998) *J. Leukocyte Biol.* **63,** 650–657
16. Medzhitov, R., and Janeway, C. A., Jr. (1997) *Cell* **91,** 295–298
17. Janeway, C. A., Jr. (1992) *Immunol. Today* **13,** 11–16
18. Williams, M. J., Rodriguez, A., Kimbrell, D. A., and Eldon, E. D. (1997) *EMBO J.* **16,** 6120–6130
19. Levashina, E. A., Langley, E., Green C., Gubb, D., Ashburner, M., Hoffmann, J. A., and Reichhart, J.-M. (1999) *Science* **285,** 1917–1919
20. Lee, W.-J., Lee, J.-D., Kravchenko, V. V., Ulevitch, R. J., and Brey, P. T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7888–7893
21. Dimopoulos, G., Richman, A., Müller, H.-M., and Kafatos, F. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11508–11513
22. Richman, A. M., Dimopoulos, G., Seeley, D., and Kafatos, F. C. (1997) *EMBO J.* **16,** 6114–6119
23. Ochiai, M., and Ashida, M. (1999) *J. Biol. Chem.* **274,** 11854–11860
24. Kang, D., Liu, G., Lundstrom, A., Gelius, E., and Steiner, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10078–10082
25. Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Van Dyck, E., Revets, H., Brys, L, Gomez, J., De Baetselier, P., and Timmermans, M. (1998) *J. Biol. Chem.* **273,** 24948–24954
26. Lee, S.-Y., Wang, R., and Soderhäll K. (2000) *J. Biol. Chem.* **275,** 1337–1343
27. Ochiai, M., and Ashida, M. (2000) *J. Biol. Chem.* **275,** 4995–5002
28. Ma, C., and Kanost, M. R. (2000) *J. Biol. Chem.* **275,** 7505–7514
29. Han, S.-J., Choi, K.-Y., Brey, P. T., and Lee, W.-J. (1998) *J. Biol. Chem.* **273,** 369–374
30. Sambrook, J., Fritsch, E. F.and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* 2nd Ed. Cold Spring Harbor Press, Plainview, NY
31. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual,* Cold Spring

Harbor Press, Plainview, NY
32. Itoh, Y., Kajita, M., Kinoh, H., Mori, H., Okada, A., and Seiki, M. (1999) *J. Biol. Chem.* **274,** 34260–34266
33. Kylsten, P., Samakovlis, C., and Hultmark, D. (1990) *EMBO J.* **9,** 217–224
34. Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broeckaert, W., Hertu, C., and Hoffmann, J. (1994) *J. Biol. Chem.* **269,** 33159–33163
35. Åsling, B., Dushay, M., and Hultmark, D. (1995) *Insect Biochem. Mol. Biol.* **25,** 511–518
36. Fyrberg, E. A., Bond, B. J., Hershey, N. D. Mixter, K. S., and Davidson, N. (1981) *Cell* **24,** 107–116
37. Yahata, N., Watanabe, T., Nakamura, Y., Yamamoto, Y., Kamimiya, S., and Tanaka, H. (1990) *Gene* (*Amst.*) **86,** 113–117
38. Seki, N., Muta, T., Oda, T., Iwaki, D., Kuma, K., Miyata, T., and Iwanaga, S. (1994) *J. Biol. Chem.* **269,** 1370–1374
39. Medzhitov, R., and Janeway, C. A., Jr. (1998) *Curr. Opin. Immunol.* **10,** 12–15
40. Pugin, J., Heumann, I. D., Tomasz, A., Kravchenko, V. V., Akamatsu, Y., Nishijima, M., Glauser, M. P., Tobias, P. S., and Ulevitch, R. J. (1994) *Immunity* **1,** 509–516
41. Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R., and Golenbock, D. (1999) *J. Immunol.* **163,** 1–5
42. Lemaitre, B., Reichhart, J.-M., and Hoffmann, J. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 14614–14619

Gram-negative Bacteria-binding Protein, a Pattern Recognition Receptor for Lipopolysaccharide and β-1,3-Glucan That Mediates the Signaling for the Induction of Innate Immune Genes in *Drosophila melanogaster* Cells

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